IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Randolph J. Noelle et al.

Application No.: 09/849,969 Confirmation No.: 1327

Filed: May 8, 2001 Art Unit: 1644

For: TREATMENT OF T CELL MEDIATED AUTOIMMUNE DISORDERS Examiner: P. Gambel

DECLARATION OF EDWARD A. CLARK, PH.D.

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Edward A. Clark, declare and state that:

1. I am a Professor in the Departments of Microbiology and Immunology at the University of Washington in Seattle. A *curriculum vitae*, which details my education, positions held, and major professional achievements, accompanies this declaration as Exhibit A.

2. I have reviewed the most recent Office Action by Examiner Gambel dated May 4, 2006, as well as U.S. Patent No. 6,592,868 issued to Lederman et al. ("Lederman") and U.S. Patent No. 5,747,037 issued to Noelle ("Noelle"). It is my understanding that the Examiner has concluded that Lederman combined with Noelle would have suggested the use of an anti-gp39 (*i.e.*, anti-CD40L) antibody to treat the T cell-mediated aspects of type I diabetes. Based upon the following, I respectfully disagree.

BACKGROUND OF T CELL VS. B CELL RESPONSES

3. T cells are divided into two broad categories, CD4+ T lymphocytes, which express the CD4 receptor ("helper" T cells) and CD8+ T lymphocytes, which express the CD8 receptor ("effector" or "cytotoxic" T cells). Immune responses are initiated by presentation of processed antigen by antigen-presenting cells (APCs) to T cells. Antigens are taken up by APCs and presented to T cells in association with either major histocompatibility complex (MHC) class I or class II molecules on the surface of the APC. CD4 binds class II MHC molecules, while CD8 binds class I MHC molecules. Peptides presented with class I molecules stimulate cytotoxic T cells to kill the cell from which the antigen was derived. In contrast, peptides presented with class II molecules stimulate helper T cells to generate further immune responses.

4. Helper T cells can be subdivided roughly into Th1 and Th2 cells based upon their profile of cytokine release. Th1 cells, also referred to as inflammatory Th1 effector cells (Janeway and Travers, Immunobiology- the Immune System in Health and Disease, Current Biology Ltd. (1994) Chapter 7), produce primarily cytokines such as interferon (IFN)-γ, which activate immune cells such as macrophages or cytotoxic T cells in the area. Th2 cells often are helper T cells for B cells, and release primarily B cell activating cytokines such as interleukin-4 (IL-4).

5. Helper T cell stimulation of B cells leads to B cell activation, proliferation and differentiation into antibody-secreting cells. Aspects of the immune response mediated by antibodies are referred to as the humoral response. Humoral responses are so characterized when the immune response can be transferred from one experimental animal to another by transfer of antigen-specific antibodies. It is the inhibition of this helper T cell stimulation of B cells and humoral responses that is disclosed in the '868 patent.

6. In humoral immune responses, binding of antibodies to antigens can target an antigen for phagocytosis, lead to complement fixation, and/or attract further inflammatory cells. These mechanisms can lead to cellular injury. An example of an antibody-mediated disease is systemic lupus erythematosus, a disease characterized by antibodies to DNA, ribonucleoproteins, and other non-organ-specific molecules (specification, page 1). Antibodies may also or alternatively block receptor function. An example of this type of humoral autoimmune response is

myasthenia gravis, a disease of progressive muscle weakness characterized by antibodies reactive to acetylcholine receptors at neuromuscular junction (specification, page 1).

7. In contrast to humoral immune responses, T cell-mediated responses are not mediated by autoantibodies and represent an immune response that is independent of B cell activation. Immune responses of this type can be transferred in experimental models by transfer of T cells as opposed to antibodies. An example of a T cell-mediated autoimmune disorder is experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis (MS), where T cells reactive to myelin cause demyelination of the brain and spinal cord. EAE can be transferred from diseased animals to healthy ones by the transfer of T cells from the diseased animals. Another example of T cell-mediated autoimmune disease is the NOD mouse, which spontaneously develops insulitis and diabetes. Diabetes can be transferred from diseased animals to healthy ones by the transfer of CD4 and CD8 T cells from the diseased animal to the healthy one (*see, e.g.,* Bendalac *et al.* (1987) Journal of Experimental Medicine 166:823 (attached as Exhibit B)). This transfer of disease does not require B cells (Bendalac *et al.* (1988) Journal of Immunology 141:2625 (attached as Exhibit C)).

8. Cellular immune responses are mediated by activated T cells specific for autoantigen presented on cell surfaces. In T cell-mediated autoimmune responses, heavy infiltration of T lymphocytes and activated macrophages are seen in affected tissues. The specification describes how induction of EAE by immunization with myelin-associated proteolipid protein (PLP) in mice leads to encephalomyelitis, characterized by "perivascular infiltrates containing lymphocytes and macrophages and the development of demyelination in the brain and spinal cord" in addition to relapsing paralysis (specification, page 9, lines 33-36). Induction of EAE by T cell transfer is a strong indicator that activated T cells are responsible for EAE development (specification, page 10, line 39 to page 11, line 2). Similarly, the induction of diabetes by T cell transfer is a strong indicator that activated T cells are responsible for the development of autoimmune type I diabetes. Exhibit B.

STATE OF THE ART IN JUNE OF 1995

Role of gp39 in T cell or Cellular Immune Responses

9. In June of 1995, one of skill in the art would not have recognized that a gp39 antagonist would have an effect on T cell-mediated disease because the mechanism of disease is independent of B cell activation and the resulting production of antibodies which was considered the primary role of gp39. It was not known in the art in June of 1995 that gp39 had any role in non-B cell immune responses, *e.g.*, CD4- and CD8-mediated cellular immunity. The disclosure of the current application provides an entirely unexpected result, specifically, that inhibition of a T cell receptor previously shown to be necessary for the disruption of T cell-B cell interactions only, is capable of modulating the progression of a disease that is non-B cell mediated.

10. For example, Mohan *et al.* (February 1995) Journal of Immunology 154:1470 (attached as Exhibit D) showed that while anti-gp39 reduced pathogenic autoantibodies *in vivo*, it did not remove T helper cells, only blocked their ability to stimulate B cell to make autoantibodies. The authors concluded that "the anti-gp39 treatment appears to block mainly the cognate, contact-dependent interaction between pathogenic Th and B cells ... ." Exhibit D, page 1479. Similarly, Biancone *et al.* (August 1995) Kidney International 48(2):458 (attached as Exhibit E) concluded that a CD40-Ig fusion protein blocked the development of membranous chronic glomerulonephritis (MGN) because "the CD40-CD40L costimulatory pathway can prevent the development of MGN by suppressing T cell-dependent *antibody production.*" Exhibit E, Abstract (emphasis added).

11. After July of 1995, studies were published that began to show a role of CD40L in regulating non-B cell immunity. For example, Wiley and Harmsen (October 1995) Journal of Immunology 155:3525 (attached as Exhibit F), reported that anti-CD40L could block CD4+ T cell-mediated resistance to infection which did not involve B cells. The finding of the specific requirement of CD40L for antigen-specific T cell priming also came after June of 1995 (*see*, *e.g.*, Grewal *et al.* (December 1995) Nature 378:617 (attached as Exhibit G)). However, even after this time it was uncertain which CD4+ T cell responses required CD40L-CD40 (*see*, *e.g.*, Oxenius et al. (1996) Journal of Experimental Medicine 183:2209 (attached as Exhibit H)).

Oxenius and his coworkers reported that "[i]n contrast to most previous reports [focused on CD40-CD40L interactions in humoral immune responses], this study examined in addition the role of CD40-CD40L interaction on Th cell activation, proliferation and effector functions. Surprisingly, neither Th cell induction nor Th cell effector functions other than cognate help for B cells were compromised by the lack of CD40- or the CD40L molecules." Exhibit H, page 2214.

12. Thus, at the time of filing the current application (June of 1995), it was not certain if gp39 (CD40L) had any role in T cell activation and effector functions.

Autoimmune Responses in Type I Diabetes

13. It was known in the art in June of 1995 that the immune response in both transplant rejections and type I diabetes had both B cell- and T cell- mediated components. In the case of spontaneous diabetes in the NOD mouse model, B cells were implicated in the development of T cell-mediated autoimmune diabetes after June of 1995, and then only as APCs, and not as effectors of humoral immunity. *See, e.g.,* Serreze *et al.* (1996) Journal of Experimental Medicine 184:2049 (attached as Exhibit I); Akashi *et al.* (1997) International Immunity 9:1159 (attached as Exhibit J).

14. The T cell mediated component of type I diabetes is the T-cell mediated destruction of beta cells. In T cell-mediated diabetic complications, autoreactive $CD8^+$ cytotoxic T cells recognize peptides from a beta cell-specific protein, bind to the beta cells, and selectively destroy these cells. This process occurs independently of any autoantibody response, because, as noted above, cytotoxic T cells are activated by APCs, which present the antigen to the T cells, and subsequently seek and destroy cells expressing the antigen. Antibodies are not necessary for this response. Therefore, a person of skill in the art would not have thought to use an anti-CD40L antibody to treat T cell-mediated effects in diabetes because completely different T cells need to be targeted. Also, it was not known in June of 1995 that CD40L was expressed on effector T cells in the pancreas.

15. It would not have been obvious to one of skill in the art in June of 1995 that an anti-CD40L antagonist or antibody could be used to treat the T cell-mediated responses of type I

diabetes. The first studies suggesting that CD40L-CD40 interactions in T cell-mediated aspects of the development of insulitis and diabetes were published in *November of 1997* (Balasa *et al.* (1997) Journal of Immunology 159:4620 (attached as Exhibit K). While the role of CD40-CD40L interactions in other B cell-mediated autoimmune diseases had been reported (*e.g.*, Mohan (Exhibit D), Balasa noted that the possibility that CD40-CD40L "participate in T cell-dependent spontaneous autoimmune disease remains untested." Exhibit K, page 4620. Until this study, the focus had been on blocking either CD28/B7 or LFA-1/ICAM interactions for the prevention of diabetes. Unlike the blockade of T cell/B cell interactions disclosed in Lederman, Balasa proposed that anti-CD40L treatment might preclude the entry of antigen-specific T cells "into the pancreas by preventing the interaction of activated T cells with vascular endothelial cells" (Exhibit K, page 4626), and prevent the T cell responses associated with diabetes. Balasa also noted that the "administration of anti-CD40L blocked the development of insulitis and diabetes, the prototypic autoimmune lesion of NOD mice. The development of islet-reactive Th1 cells is impaired without concomitant switch to Th2 cells." Exhibit K, page 4624.

16. Diabetic autoimmune disease may involve humoral-mediated and/or cell-mediated tissue damage, but the mechanisms of damage are distinct. Diabetic tissue damage or dysfunction mediated by B cells would have been considered treatable by application of gp39 or CD40L antagonists in June of 1995. However, for the reasons set forth above, destruction of beta cells by cytotoxic T cells, and inflammation caused by T cell-mediated activation of other immune cells such as macrophages, would not be considered treatable or preventable by administration of CD40L antagonists at that time. Thus, one of skill in the art would not expect to be able to treat this aspect of autoimmune response using the disclosure in Lederman, which relates to only antagonists of the helper T cell-B cell interaction.

THE NOELLE '037 PATENT

17. The disclosure of the Noelle '037 patent would not lead a person of skill in the art to treat diabetes with anti-gp39 antagonists, either alone or in combination with other prior art. Noelle does teach a method for inducing antigen-specific T cell tolerance and a means to block allogeneic T cell responses as measured by graft versus host disease (GVHD) using anti-gp39 monoclonal antibodies. One skilled in the art would have thought that this treatment might be

pertinent for the treatment of diabetes to the extent that the method in Noelle involves pancreatic allografts. However, this would not suggest that the underlying disease of diabetes could be directly treated with an anti-gp39 antagonist. Nor was it known in June of 1995 that pancreatic allograft rejection could be treated with anti-CD40L antagonists. This finding was published in October of 1995 (Parker *et al.* (October 1995) PNAS 92:9560 (attached as Exhibit L)). In short, the Noelle '037 patent concerns the induction of antigen-specific T cell tolerance that would applicable to allogeneic transplantation or autoimmune disease where the autoantigens are clearly defined.

THE LEDERMAN '868 PATENT

18. The Lederman '868 patent states that the "*method of inhibiting B cell activation* is valuable in a new and useful method for inhibiting the immune response of an animal." (Lederman, col. 11, ll. 18-20 (emphasis added)). One embodiment of this "method of inhibiting B cell activation" is inhibiting the autoimmune response in an animal suffering from autoimmune disease." (Lederman, col. 11, ll. 30-31). Lederman then gives examples of autoimmune diseases which include diabetes mellitus. However, nothing in the Lederman '868 patent teaches or suggests that the disclosed method can be used to inhibit T cell activation, or treat the T cell-mediated aspects of autoimmune disease.

19. The Lederman '868 patent describes the disclosed method strictly in terms of B cell activation. This disclosure would not teach or suggest the present invention because the CD40L antagonists are taught to target helper T cell/ B cell interaction, not T cell-mediated immunological responses. For example, Lederman presents data in Figure 11 showing that CD40L/gp39 is not expressed on CD8+ T cells. Moreover, Lederman states that "[t]he monoclonal antibody described and claimed herein binds to T cells which are interacting with B cells in the germinal centers of lymph nodes and not to other T cells." (Lederman, col. 6, ll. 65-67). Lederman also states that "[f]or the purposes of this invention, 'activated T cells' are T cells capable of providing T cell helper function to resting B cells." (Lederman, col. 7, ll. 6-8). The cell line used in Lederman, D1.1 is described as a T cell "capable of constitutively providing contact-dependent helper function to B cells." (Lederman, col. 9, ll. 4-9). These statements indicate that the Lederman '868 patent does not concern T cell helper function for non-B cells,

but rather only is concerned with T cells providing helper function of B cells, particularly those B cells within germinal centers.

20. Additionally, the Lederman '868 patent does not even teach a person of skill in the art to treat the B cell-mediated diseases because their model systems are flawed. There are no data anywhere in the '868 patent showing the effect of monoclonal antibody 5c8 on autoimmune responses or autoimmune diseases. Also, there are no data showing the effect of normal human T cells expressing what is called T-BAM on an antigen-specific immune response *in vitro* or *in vivo*.

21. The Lederman '868 patent does not teach anything about the treatment of autoimmune disease because it uses a human T cell line, Jurkat, which proliferates continuously in culture. One of skill in the art would not have used Jurkat T cells to study the role of monoclonal antibody 5c8 on autoimmune responses or autoimmune diseases because Jurkat is a transformed T cell leukemia line and cannot be used to induce antigen-specific responses *in vitro* or *in vivo*. At the time of the present application, one of skill in the art would have recognized that signaling a human T cell line, Jurkat, with an anti-CD3 monoclonal antibody, was not the same as signaling with an antigen.

22. The '868 patentees simply used the Jurkat cells as a convenient means to study B cell activation. This is not the same as addressing the role of CD40-CD40L in autoimmune responses since antigen-specific responses were not examined. Furthermore, it was known that resting T cells do not express CD40L and that the expression of CD40L is regulated on T cells. For example, T cell receptor signaling was known to induce the expression of CD40L on T cells and that CD28 signaling sustains CD40L expression on T cells (*see, e.g.*, Clark and Ledbetter (1994) Nature 367:425 (attached as Exhibit M). Thus, a Jurkat cell subline (D1.1), which is a continuously dividing T cell line and unlike normal T cells, constitutively expresses high levels of CD40L, to activate B cells, was not a good model for the interactions that could occur between normal T cells and normal B cells.

23. In the experiments where normal T cells are used in Lederman, a tumor promoting agent, PMA, and PHA, (Lederman, Example 6, col. 23, ll. 38-53), a combination of phorbol dibutyrate

(PDB) and anti-CD3 (Lederman, Example 7, col. 23, l. 55- col. 24, l. 26)[1], or pokeweed mitogen (PWM) (Lederman, Example 7, col. 24, ll. 27-59) is used to activate the T cells. There is no specific antigen used in any example in the Lederman '868 patent. Furthermore, Lederman does not demonstrate that CD40L is induced on normal T cells by specific antigens.

24. The '868 patent does not provide evidence showing that the 5c8 anti-CD40L monoclonal antibody binds to cells other than human T cells stimulated with non-physiologic stimuli and the Jurkat human T cell line, and certainly provides no evidence that the 5c8 antibody can affect an autoimmune disease in an animal including humans. There are no functional data in Lederman using T cells activated with physiologic stimuli, *i.e.*, antigen, and no data assessing the role of an anti-CD40L antibody *in vivo* which would be essential to know if the antibody could inhibit autoimmune disease.

I declare further that statements made in this declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: 6 Nov 2006

Edward A. Clark, Ph.D.

[1] In this experiment, the T cells were killed by fixation prior to being put in contact with the B cells.

CURRICULUM VITAE - EDWARD ALAN CLARK

Summer 2006

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PERSONAL

Birth:	September 3, 1947, Long Beach, California
Citizenship:	USA
Marital status:	Married to Barbara L. Clark, M.D., nee Siemens
Children:	Naomi (30), Sashya (28), Cary (24) and Amelia (9)

DEGREES HELD

B.A.	University of California Los Angeles (UCLA)	1969	Psychology/Zoology
Ph.D.	UCLA	1977	Microbiology/Immunology

RESEARCH AND PROFESSIONAL EXPERIENCE

2001-**present**	Professor, Immunology, University of Washington
1990-**present**	Professor, Microbiology, University of Washington
1979-**present**	Core Staff Scientist, National Primate Research Center, University of Washington, Seattle, WA (with two-year absence, 1982-84)
2002-**present**	Head, Hematologic Resources Division, National Primate Research Center, University of Washington
1996-2002	Head, Virology and Immunology Division, Regional Primate Research Center, University of Washington
1991-2001	Adjunct Professor, Immunology, University of Washington
1989-1998	Program Director, Lymphocyte Activation Group, University of Washington
1988-2002	Member, Center for AIDS Research, University of Washington
1987	Foreign Research Scholar, Division of Immunology, Institute of Molecular and Cellular Biology, Osaka University, Osaka, Japan (with Tadamitsu Kishimoto)
1986-1996	Director, Immunology/AIDS Flow Cytometry Facility, University of Washington
1984-90	Associate Professor, Microbiology and Immunology, University of Washington
1981-84	Founder and Senior Scientist, Genetic Systems Corporation, Seattle, WA
1980-90	Affiliate, Fred Hutchinson Cancer Research Center, Seattle, WA
1979-84	Assistant Professor, Genetics, University of Washington, Seattle, WA
1977-79	Honorary Staff Research Assistant, Department of Zoology, University College, London (with N. Avrion Mitchison)
1974-77	Postgraduate Research Assistant, Department of Microbiology and Immunology, UCLA (with William H. Hildemann)
1970-74	Staff Research Associate and Renal Transplant Coordinator, Department of Surgery, UCLA (with Paul I. Terasaki)
1968-69	Research Assistant, Department of Surgery, UCLA

HONORS

2006-**present**, Member, Henry Kunkel Society
NIAID MERIT award, July 2004 for grant AI444257.
Science Watch Most Highly Cited Authors in Immunology 1981-2001, November 2001
Science Watch Most Highly Cited Authors in Immunology 1990-94, No. 15, May 1995
Japanese Ministry of Education (Mombusho), Foreign Research Scholar, Osaka University, 1987
Edna A. Old Memorial Fellow of the Cancer Research Institute of New York, 1977-1979
Inter-Science Research Foundation Graduate Student Research Prize, 1977
University of California Regents Scholar, 1965-1969

MAJOR RESEARCH INTERESTS

B lymphocytes: Activation and fate, immunobiology, interactions with other cells, cancer
Dendritic cells: immunobiology, C-type lectin receptors, signaling, interaction with pathogens
Molecular and cellular mechanisms of germinal center formation

CURRENT RESEARCH SUPPORT

Principal Investigator: "FDCR1 and follicular dendritic cell signaling pathways, " **NIAID MERIT AWARD** R37AI44257-07, Merit Award began in year 06, July 2004-June 2009, with non-competitive renewal possible for additional 5 years.

Principal Investigator: "Structure/function human B cell differentiation antigens," **NIGMS** R01 GM37905-19, January 1986-March 2008.

Principal Investigator: "Dendritic cell associated C-type lectins" **NIAID** 1R01AI52203-04, April 2003-March 2008.

Principal Investigator: "Dendritic cells, mucosal immunity and C-type lectins', **NIDCR** R01 DE016381-02, May 2005-April 2010.

Core Staff Scientist: Regional Primate Research Center, **NCRR** P51 RR00166-45 July 2002-June 2007; head of 'Dendritic Cell Biology Core'.

Dr. Clark has been an active member of an Immunology Training Grant for 17 years from **NCI**.

PROFESSIONAL AND EDITORIAL AFFILIATIONS

American Association of Immunologists, 1979-**present**
American Association for the Advancement of Science, 1979-**present**
Editorial Board, *Human Immunology*, 1999-**present** (2003-present called *Molecular and Structural Immunology*)
Founder and Chair, Scientific Advisory Board, Trubion Pharmaceuticals, Inc., Seattle, WA, 2001-**present**
Transmitting Editor, *International Immunology*, 2001-2006
Scientific Advisor, Xcyte Therapeutics Corporation, Seattle, WA, 1997-2006
Advisory Board, *Tissue Antigens*, 1990-2004
Deputy Editor, *Cellular Immunology*, 1996-2001
Editorial Board, *Natural Immunity and Cell Growth Regulation*, 1983-1986
Council, Midwinter Conference for Immunology, 1981-1985, 1992-1997
Scientific Advisor, Ultradiagnostics Corporation, Seattle, WA, 1985-1987

Editorial Board, *Journal of Clinical Immunology*, 1988-1993
Section Editor, *Journal of Immunology*, 1992-1995

REVIEWING/EXTRAMURAL SCIENTIFIC SERVICE

Reviewer, American Cancer Society Institutional Research Grant, University of Washington, 1981-1987, 1995-2003.
Reviewer, Biomedical Research Support Grants (BRSG), University of Washington 1990, 1991; Royal Research Fund grants, 1994-2004
NIH Reviewers Reserve, 1994-**present** (various reviews and site visits), e.g., ALY Study Section Feb 2004; CMIA Review Group, 2005, 2006, P01 grant review Mar 2004, Special Emphasis Panels, 2003-**present**
Member, NIH Immunological Sciences Special Study Section, 2001-2005
Member, National Research Council (NRC) Advisory Committee on Biomedical Workshops and Laboratory Courses in Central/Eastern Europe, 2000-2004
Reviewer, National American Cancer Society Cancer Immunology Review Group, 1996-2001; Chairman, Leukemia, Immunology and Blood Cell Development Review Committee, 2000-2001
External reviewer, Tufts University Immunology Program Review, May 2000
Ad Hoc Reviewer, Immunology Group, National American Cancer Society, 1990, 1996
Ad Hoc Reviewer, NIH Study Sections: Experimental Immunology (1990, 1995) and Allergy and Immunology (2003)
Member, NIH Experimental Immunology Study Section, 1990-1994
Reviewer, Marion Merrell Dow Foundation Fellowships, 1992
Reviewer, Intramural Program, Laboratory or Immunology, National Institute of Dental Research, 1994
Review periodically NSF, Veteran's Administration, New Zealand or United Kingdom MRC grants
Review periodically manuscripts from *Nature, Science, Proceedings of the National Academy of Sciences, Immunity, Journal of Experimental Medicine, Journal of Cell Biology, Journal of Immunology, Journal of Clinical Investigation, Blood, EMBO Journal, International Immunology, Molecular and Cellular Biology, Current Biology, AIDS, Human Immunology, Scandinavian Journal of Immunology* and *Tissue Antigens*.

SELECTED LECTURES AND MEETINGS

Symposium Speaker, Mol. Cell Biology Symposium, Oslo, Norway: 1986
Symposium Speaker, Amer. Soc. Histocompat. Immunogenetics, New Orleans: 1986
Lecture, Department of Microbiology, University of Hong Kong, Hong Kong: 1987
Lecture, Department of Microbiology, School of Veterinary Medicine, Bogor, Indonesia: 1987
Speaker, Dept. Microbiology, Sun Ya Sen College of Medical Sciences, Guangzhou, China: 1987
Lecture, Department of Immunology, Nagoya University School of Medicine, Nagoya, Japan: 1987
Lecture, Department of Immunology, Tokyo University, Tokyo, Japan: 1987
Symposium Speaker, Nonhuman Primate Models in Biomedicine, Tokyo, Japan: 1987
Lecture, Department of Microbiology and Immunology, University of Oregon, Portland: 1988
Lecture, Department of Microbiology, University of California, Irvine: 1989
Plenary Lecture, Fourth International Leukocyte Differentiation Antigen Workshop, Vienna: 1989
Workshop Speaker, New Advances in Flow Cytometry, San Francisco: 1989
Workshop Speaker, FASEB Meeting, New Orleans: 1989
Workshop Chairman, Seventh International Immunology Congress, Berlin: 1989
Speaker, Lymphocyte Activation and Development, U.S. - Japan Exchange Program, National Cancer Institute, Bethesda, MD: February, 1990; Osaka, Japan, November 1990
Workshop Chairman, B Cell Signaling, UCLA Symposium on B Cell Development, Park City, UT, 1990
Symposium Chairman, Nonhuman Primate Models in AIDS, New Orleans, 1990
Lecture, Department of Biology, University of California, San Diego, 1991
Session Chairman and Speaker, FASEB Summer Research Conference, Vermont, 1991
Minisymposium Speaker, International AIDS Congress, Florence, Italy, 1991
Lectures at Stanford University, University of California at San Francisco and UC at Berkeley, 1992

Lecture, University of Rochester, NY, 1992
Cochairman, Minisymposium "B Lymphocytes in Health and Disease" FASEB, Anaheim, CA, 1992
Lecture, Institute for Molecular Genetics, Czechoslovak Academy of Sciences, Prague, 1992
Lecture, Department of Pathology, New York University, NY, 1992
Speaker, Midwinter Conference in Immunology, Asilomar, CA, 1993
Co-organizer and Symposium Speaker, "B Cell Immunobiology and Human Disease", Keystone Symposium, Taos, NM, 1993
Lecture, University of Virginia, Charlottesville, VA, 1993
Speaker, Congreso de la Asociacion Latinoamericana de Immunologia (ALAI), Santiago Chile, 1993
Lecture, Department of Microbiology, Boston University, MA, 1993
Lecture, Moffitt Cancer Center, Tampa, FL, 1993
Symposium Speaker, Nonhuman Primate Models in AIDS, Madison, WI, 1993
Symposium Speaker, 8th International Lymphokine Workshop/4th International Workshop on Cytokines, Osaka, Japan, 1993
Speaker, Inter. Workshop on Chronic Lymphocytic Leukemia, Wright City, MO, 1993
Symposium Speaker, 5th Int. Conference on Lymphocyte Activation and Immune Regulation, Newport Beach, CA, 1994
Lecture, Jefferson Cancer Institute, Philadelphia, PA, 1994
Lecture, Scripps Clinic and Research Foundation, La Jolla, CA, 1994
Lecture, Department of Laboratory Medicine, University of Minnesota, Minneapolis, MN, 1994
Speaker and Session Co-chair, Third International Symposium on Dendritic Cells in Fundamental and Clinical Immunology, Annecy, France, 1994
Speaker, T Cells and Cytokines in Health and Disease Workshop, Oxford, England, 1994
Lecture, Cambridge University, England, 1994
Speaker, "Advances in Bone Marrow Transplantation and Treatment of Complications", Newport Beach, CA, 1994
Speaker, American Association of Cancer Research Conference "Translational Research in Cancer: New Opportunities for Progress", Asheville, NC, 1994
Lecture, University of Toronto, Toronto, Canada, 1995
Lecture, La Jolla Institute of Allergy and Immunology, La Jolla, CA, 1995
Lecture, University of Chicago, Chicago, IL, 1995
Lecture, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA, 1995
Lecture, Sunley Research Centre, London, England, 1995
Speaker, 42nd University of Michigan Cancer Retreat, Battle Creek, MI, 1995
Speaker and Workshop Chairman, European Research Conference, "B Cells in Normal and Disease State: The Molecular Basis of Human B Cell Disorders', Lunteren, The Netherlands, 1995
Symposium Speaker, Nonhuman Primate Models in AIDS, Monterey, CA, 1995
Workshop Chairman, American Society of Hematology, Seattle, WA, 1995
Symposium Speaker, Latin America Immunology Congress, Zacatecas, Mexico, 1996.
Lectures, Human Genome Sciences, Washington, DC; Cornell Medical Center, New York, NY; North Shore University Hospital, Manhasset, NY; Cleveland Clinic, OH, March 1996
Lecture, Washington University, St. Louis, MO, 1996
Plenary Symposium Speaker, British Society of Immunology, The Biochemical Society, Harrowgate, United Kingdom, 1996
Lecture, University of California, San Francisco, CA, 1997
Speaker, 7th International Workshop on CLL, Crete, Greece, 1997
Speaker, 9th International Symposium on the Immunobiology of Proteins and Peptides, Whistler, BC, Canada, 1997
Participant, AIDS Vaccine Research Committee workshop on "Development of an effective immunity to HIV", Redwood City, CA, 1997
Lee Bartell Visiting Professor, University of California, San Diego, 1997
Lecture, Yale University, New Haven, CN, 1998
Workshop Speaker, British Society of Immunology, Harrowgate, United Kingdom, 1998
Lecture, Sunley Research Centre, London, England, 1998

Organizer and Speaker, Keystone Symposium, "B Lymphocyte Biology and Disease", Taos, NM, 1999
Lecture, Seattle Biomedical Research Institute, Seattle, 1999
Speaker, "From the Laboratory to the Clinic", Trinity College, Oxford, September 1999
Speaker, 8th International Workshop on CLL, Paris, October 1999
Lectures, Kansai Medical University and Osaka University, November 1999
Interview about immunology, NHK public radio, Tokyo, Japan, December 1999
Lecture, MD Anderson Cancer Center, Houston, January 2000
Lecture, Pacific Northwest Research Institute, Seattle, April 2000
Block Symposium Co-chair, "B lymphocyte signaling II- cell fate, decisions and immune response", American Association of Immunology annual meeting, Seattle, May 2000
Chairman, B cell Section, Leucocyte Typing VII Workshop, Harrowgate, UK, June 2000
Speaker, FASEB Summer Conference on Lymphocyte Activation, July 2000
Speaker, Keystone Symposium, "Dendritic Cells: Interfaces with Immunobiology and Medicine", Taos, NM, March 2001
Chairman, Workshop on coreceptors and B cell activation, Keystone Symposium, "B Lymphocyte Biology and Disease", Snowbird, UT, April 2001
Speaker, The Antibody Club, London, June 2001
Interview about Buddhist perspective on 9/11 attack, KUOW Radio, Seattle, 9/14/01
Speaker, University of Minnesota Cancer Center, Minneapolis, MN, October 2001
Speaker, New Advances in Biologic Therapy: Applications in Oncology and Immunology, Maui, Hawaii, January 2002
Speaker, 9th International Workshop on CLL, San Diego, CA, March 2002
Speaker, United Kingdom CLL Forum, London, May 2002
Lecture, University of Southampton, Southampton, UK, May 2002
Speaker, Lymphocyte Signal Transduction Workshop, Santorini, Greece, October 2002
Speaker and Workshop Chairman, Keystone Symposium 'B Lymphocyte Biology and Human Disease', Keystone, CO, January 2003
Symposium Speaker, 10th IW-CLL Workshop, Stressa, Italy, October 2003
Speaker, NIH, Bethesda, MD, November 2003
Speaker, American Association for the Advancement of Sciences, Innate Immunity Symposium, Seattle, February 2004
Lecture, Scripps Clinic and Research Foundation, La Jolla, CA, October 2004
Lecture, Immune Tolerance Network, Chicago, October 2004
Speaker, 6th Conference on Advances in Targeted Therapies, Juan les Pins, France, April 2005
Speaker 5th Parnas Conference 'Molecular Mechanisms of Cellular Signaling', Kyiv, Ukraine, April 2005
Speaker, Satelite Symposium 'Molecular Aspects of Carcino- and Leukemogenesis, Kavetsky Institute of Experimental Pathology, Radiology and Oncology, Kyiv, Ukraine April 2005
Lectures, University of California, San Diego and La Jolla Institute for Allergy and Immunology, June 2005
Moderator, US/Japan Board Symposium, Seattle, WA, July 2005
Speaker, Henry Kunkel Society Symposium, Cambridge, United Kingdom, August 2005
Symposium Speaker, 7th Latin American Immunology Congress, Cordoba, Argentina, October 2005
Graduate Student Invited Lecture, University of Toronto, November 2005
Lecture, University of Pennsylvania, April 2006
Lecture, University of California, Irvine, June 2006

TEACHING EXPERIENCE

University of Washington

Undergraduate:

Introduction to Immunology (Microbiology/Immunology 441, or Immunology 441): Coordinator, 1985-1992 (coordinated and gave most lectures in undergraduate course, approx. 100-150 students); lecturer, 1994-1999
Topics in Immunology (Microbiology 447): 1985-90
Methods in Immunology (Microbiology 448): 1985, 1986, 1988

Graduate:

Topics in Immunology (Immunology 534): Course Organizer, 1994-2003, Faculty session leader 2005-**present**
Host Defense to Cancer and Infection, Course Organizer (Immunology 533): 2002-**present** (changed in 2005 to Host Defense to Cancer)
Host Defense to Infection (Immunology 535): Course organizer to begin 2006.
Lectures, Developmental Immunology (Immunology 533 then 532): 1995-1998, 2002-2004
Immunogenetics (Genetics 571): 1980, 1982, 1983
Molecular and Cellular Immunology (Microbiology 572): 1986, 1988
Seminars in Immunology (Microbiology 573): 1986, 1988, 1989
Immunology Journal Club (Microbiology 551): 1989-90
Lectures, Bacterial Pathogenesis (Microbiology 553): 1993
Lectures, Viral Pathogenesis (Microbiology 540, 552): 1993, 1996
Lectures, Advanced Immunology (Immunology 532), 1996

Virupa Educational Institute

Introduction to Buddhism: 1997-2005
Yoga for Meditators: 1999
Introduction to Tibetan Buddhism: 2004
Buddhism and Science: Compassionate Skepticism: 2004

UNIVERSITY COMMITTEES/ADMINISTRATION

Member, Immunology, Retreat Committee, 2004-**present** (Chair, 2006)
Member, Microbiology, Corporate and Industrial Sponsorships Committee, 2000-**present**
Member, Microbiology, Appointments and Promotions Committee, 2000-**present**
Member, Sandra L. Clark Immunology Education Fund, 1992-**present**
Member, Medical School Admissions Committee, 1993-**present**
Member, Microbiology, Ordal Award Committee, 1997-2001
Member, Immunology Training Grant Advisory Committee, 1985-2005
Member, Immunology, Oral Qualifying Exam Committee, 1993-2002, Chair, 2002
Member, Cell Sorter Committee, 1986-89
Member, Microbiology Graduate Admissions Committee, 1986-1990
Chairman, Genetic Systems Health and Safety Committee, 1981-1984
Member, Primate Center Administrative Advisory Committee, 1988-1995
Member, Admissions Committee, Medical Scientist Training Program 1989-1993
Program Director, Lymphocyte Activation Group, 1989-1998
Member, Deans Scientific Affairs Faculty Council, School of Medicine, 1990-92
Member, Molecular and Cellular Biology Training Grant Selection Committee, 1993-1995
Member, Faculty Senate, 1995-1997

PUBLIC SERVICE

Volunteer, Bailey Bouchay House (AIDS Hospice), Seattle, 1993-1995

Member, Board of Directors, Sakya Monastery of Tibetan Buddhism, Seattle, 1999-2001
Trustee, Virupa Ecumenical (Educational) Institute, Seattle, 2000-2001
Organizer, Oxfam Hunger Banquet, Seattle, November 2001
Member, Board of Directors, Virupa Educational Institute, Seattle, 2002
Member, Board of Directors, Toys on a Mission, Seattle, 2004-2005

VISITING SCIENTISTS

Keiji Terao, Ph.D., Japan, 1986
Anthony Pezzutto, M.D., West Germany, 1986, 1988
David Andrew, Ph.D, United Kingdom, 1988
Elizabeth Genot, Ph.D., France, 1991, 1992
Yu Sun, M.S., Peoples Republic of China, 1995-1999
Svitlana V. Mikhalap, Ph.D., Ukraine, 1998, 1999, 2004, 2005
Adriana Gruppi, Ph.D., Argentina, 2002-2003
Svetlana Sidorenko, Ph.D., Ukraine, 1991-1996, 1996-2005
Martha Hayden-Ledbetter, Ph.D., USA, 1999, 2006-present, Primate Center, targeting dendritic cells and antibody engineering

POSTDOCTORAL FELLOWS/RESEARCH FACULTY

Takashi Yokochi, M.D., Japan, 1980-1982
Jeannine M. Durdik, Ph.D., USA, 1981-1982
Lakshmi K. (Bulusu) Gaur, Ph.D., India, 1983-1988
Thomas P. Loughran, Jr., M.D., USA, 1984-1985
Lynn (Massman) Rose, Ph.D., 1983-1986
Alexa Cheerva, M.D., USA, 1985-1986
Mary A. Valentine, Ph.D., USA, 1985-1987 (affiliated as a Res. Assist. Prof., 1987-1994)
Thomas B. Barrett, M.D., Ph.D., USA, 1988-1990
Peter J.L. Lane, M.D., Ph.D., Zimbabwe/United Kingdom, 1988-1990
Fiona M. McConnell, Ph.D., Australia, 1989-1990
Shin-Ichiro Kashiwamura, Ph.D., Japan, 1989-1991
Corinne Leprince, Ph.D., France, 1990-1992
Inger Axberg, Ph.D., Sweden, 1990-1993
Patricia Polacino (Firpo), Ph.D., Argentina, 1990-1994
Ingolf Berberich, Ph.D., Germany, 1993-1995
Kristin Brevik Anderson, Ph.D., Norway, 1993-1995
Friederike Siebelt, Ph.D., Germany, 1994-1995
Stephen J. Klaus, Ph.D., USA, 1991-1996
Che-Leung Law. Ph.D., Hong Kong, 1990-1995 (affiliated as a Res. Assistant Prof. 1995-1998)
Lesya Pinchuk, M.D., Ukraine, 1992-1997
Geraldine Grouard, Ph.D., France, 1997-1998
Alexandra Aicher, M.D., Germany, 1997-1999
Hongxia He, M.D., China, 1999-2000
Aaron Marshall, Ph.D., Canada, 1997-2000
Theodore J. Yun, Ph.D., Canada, 1996-2000
Kevin Otipoby, Ph.D., USA, 2000-2001
Eric Olsen, Ph.D., USA, 2000-2001
Kambiz Yaraei, M.D., Ph.D., Iran, 2002
Aimee Kohn, M.D., Ph.D., USA, 2003
Elizabeth Ryan, Ph.D., Ireland, 2000-2003

Helen Floyd, Ph.D., United Kingdom, 2001-2004
Jonathan Graves, Ph.D., United Kingdom, Acting Asst. Professor, Immunology, 1998-2001, Research Asst. Professor, 2001-2004
Hiroaki Niiro, M.D., Japan, 1998-2004
Thomas Yankee, Pharm. D., Ph.D., USA, 1999-2005.
Asa Bengtsson, Ph.D., Sweden, 2001-2005
Andrew Craxton, Ph.D., United Kingdom, 1995-2000, Research Asst. Professor, Microbiology, 2000-2006,
Daniela Giordano, Ph.D., Italy, 2002-present, Instructor of Immunology since 2004, regulation of dendritic cell migration and survival
Lorna Santos, Ph.D., Canada, 2003-present, dendritic cell-B cell interactions
Chie Watenabe, Ph.D., Japan, 2003-present, caspase-6 and B cells
Kevin Chen, Ph.D., Taiwan, 2003-present, Instructor of Immunology, Primate Center/vaccines
Grant Hughes, M.D., 2004-present, dendritic cells and autoimmune diseases

GRADUATE STUDENTS

Richard D. Holly, M.S., USA, 1980-1982
Raul M. Torres, Ph.D., Peru/USA, 1986-1992
Bijan Afar, D.D.S., M.S., Iran/USA, 1987-1990
Leslie A. Knapp, Ph.D., USA, 1992-1994, UCLA transfer student
Raymond T. Doty, Ph.D., USA, 1990-1997
Sasha Solow, Denmark, 1997-1999, master's project from Denmark
Kevin Otipoby, USA, 1995-2000
Cara Lerner, USA, 1999, rotation student
Wu Li, Peoples Republic of China, 2000, rotation student
Erica Andersson-Nissen, 2001, rotation student
Aimin Jiang, Peoples Republic of China, 1997-2002
Scott Canavera, rotation student, fall 2001
Jared Lopez, rotation student, Fall 2002
David Soper, rotation student, Winter 2003
Anna Pironne, 2004-2006, M.S., Immunology
Deborah Hendricks, rotation student, Fall 2005
Deanna Gilbertson, rotation student, Spring 2006
Takahiro Chino, D.D.S., Japan, 2001-present, Oral Health Center and Oral Biology
Sabrina Richards, USA, 2003-present, B cell signaling
Shinju Kasahara, Japan, 2004-present, dendritic cell-associated lectins
Daphne Ma, Hong Kong, 2006-present, dendritic cell-B cell interactions

CURRENT Ph.D. COMMITTEES

Bryan Carson, Immunology, 2001-2006
Mingyi Sun, Immunology, 2002-
Alexandra Few, Phamacology, 2004-
Sarah Andrews, Immunology, 2005-
Benjamin Buelow, Immunology MSTP, 2006-

SELECT UNDERGRADUATE STUDENTS

Dario M. Magaletti, B.S., Italy, 1992-1994, became technician in lab
Fion Lau, Hong Kong, 1999-2001, graduate school
Ilias Caralopoulos. 2002-2004, medical school

TECHNICAL STAFF

Geraldine L. Shu, M.S., USA, 1983-present, Research Scientist III, CD40/B cell markers
Kevin E. Draves, B.S., USA, 1983-present, Res. Tech. III, dendritic cell-B cell interactions
Hiroko Mihara, B.A., USA, Lab. Tech. II, 1985-present, Primate Center
Chang Li, M.S., China, Research Scientist II, 2003-present, regulation of dendritic cells
Sylvia Chien, M.S., China, Research Scientist III, 2006-present, dendritic cell targeting
Jerrilyn Sturge, M.S., USA, 1979-1982, biotechnology
Nancy T. Windsor, M.S., USA, 1980-1982, graduate school, Ph.D. in microbiology
Edna Dickinson, B.A., USA, 1980-1983, biotechnology, ICOS Corporation
Nicki Rollins, B.S., USA, 1982-1984, biotechnology
Raul Torres, B.A., Peru , 1985-1986, graduate school, Ph.D in immunology, Asst. Professor, Uni. Colorado, Denver
Michael J. Gale, B.S., USA, 1986-1990, graduate school, Ph.D. in pathobiology, Assoc. Professor, Uni. Texas, Dallas
Mark S. Scheibel, Ph.D., USA, 1989-1992, biotechnology
Joan Gibbon, B.A., USA, 1988-1990, graduate school
Mary Touma, B.A., Palastine, 1989-1995, retired
Annie Liang, M.S., Peoples Republic of China, 1991-1994, moved
Karen A. (Licciardi) Chandran , B.S., USA, 1992-1996, moved
Sandra Aung, B.A., USA, 1994-1995, graduate school, Ph.D. immunology, postdoc. Scripps Res. Institute
Jason P. Norsen, B.S., USA, 1995, medical school
Kathy Snyder, M.S., USA, 1986-1996, moved out of town
Maria K. Ewings, B.S., USA, 1996-2000, medical school, M.D., 2004
Kenneth Garbutt, B.A., USA, Res. Tech III, 2000-2002, new job
Reid Mukai, B.A., USA/Hawaii, Animal Tech. II, 2001-2003, new job
Dario M. Magaletti, B.S., Italy, Res. Scientist III, 1994-2005, retired

SCIENTIFIC PUBLICATIONS

EDWARD ALAN CLARK, Ph.D.

Summer 2006: 299 publications including 197 peer-reviewed articles, 53 non-peer-reviewed articles, 40 reviews/commentaries, 3 patents, 1 book and 5 other publications. *** = Not listed in Pub-Med. Approximate citations are in ( ).

Peer-Reviewed Articles

1. **Clark, E. A.**, Opelz, G., Mickey, M. R., and Terasaki, P. I. Evaluation of Belzer and Collins kidney preservation methods. *Lancet 1*:361-364, 1973. (16)

2. **Clark, E. A.**, Terasaki, P. I., Opelz, G., and Mickey, M. R. Cadaver kidney transplant failures at one month. *N. Eng. J. Med. 291*:1099-1102, 1974. (111)

3. **Clark, E. A.** and Hildemann, W. H. Genetics of graft-versus-host reactions. I. Production of splenomegaly and mortality in mice disparate in H-2I subregions. *Immunogenetics 4*: 281-293, 1977. (21) ***

4. **Clark, E. A.** and Hildemann, W. H. Genetics of graft-versus-host reactions. II. Interallelic effects and regulation of GVHR by anti-recipient antibodies. *Immunogenetics 5*: 309-324, 1977. (16) ***

5. Harmon, R. C., **Clark, E. A.**, O'Toole, C., and Wicker, L. S. Resistance of H-2 heterozygous mice to parental tumors. I. Hybrid resistance and natural cytotoxicity to EL-4 are controlled by the H-2D-Hh-l region. *Immunogenetics 4*: 601-607, 1977. (86)

6. **Clark, E. A.**, Harmon, R. C., and Wicker, L. S. Resistance of H-2 heterozygous mice to parental tumors. II. Characterization of Hh-l-controlled hybrid resistance to syngeneic fibrosarcomas and the EL-4 lymphoma. *J. Immunol. 119*: 648-656, 1977. (34)

7. Harmon, R. C., **Clark, E. A.**, Reddy, A. L., Hildemann, W. H., and Mullen, Y. S. Immunity to MCA-induced rat sarcomas: analysis of *in vivo* and *in vitro* results. *Inter. J. Cancer 20*: 748-758, 1977. (6)

8. Howes, E. M., **Clark, E. A.**, Smith, E., and Mitchison, N. A. Mouse hybrid cell lines produce antibodies to herpes simplex virus type 1. *J. Gen. Virol. 44*: 81-87, 1979. (28)

9. **Clark, E. A.**, Russell, P. H., Egghart, M., and Horton, M. A. Characteristics and genetic control of NK cell-mediated cytotoxicity activated by naturally acquired infection in the mouse. *Inter. J. Cancer 24*: 688-699, 1979. (69)

10. Lake, P., **Clark, E. A.**, Khorshidi, M., and Sunshine, G. H. Production and characterization of monoclonal cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. *Europ. J. Immunol. 9*: 875-886, 1979. (235)

11. Shortman, K., Linthicum, D. S., Battye, F. L., Goldschneider, I., Liabeuf, A., Goldstein, P., **Clark, E. A.**, and Lake, P. Cytotoxic and fluorescent assays for thymocyte subpopulations differing in surface Thy-1 level. *Cell Biophysics 1*: 255-270, 1979. (15)

12. Durdik, J. M., Beck, B. N., **Clark, E. A.**, and Henney, C. S. Characterization of lymphoma cell variant selectively resistant to natural killer cells. *J. Immunol. 125*: 683-688, 1980. (66)

13. **Clark, E. A.** and Holly, R. D. Activation of natural killer (NK) cells in vivo with H-2 and non H-2 alloantigens. *Immunogenetics 12*: 221-235, 1981. (25)

14. **Clark, E. A.** and Sturge, J. C. Phylogeny of NK cell reactivity against human and nonhuman primate lymphoblastoid cell lines. Evolving and conserved target antigens. *J. Immunol. 126:* 969-974, 1981. (23)

15. **Clark, E. A.**, Shultz, L. D., and Pollack, S. B. Mutations in mice that influence natural killer (NK) cell activity. *Immunogenetics 12*: 601-613, 1981. (81)

16. Engel, D., **Clark, E. A.**, Held, L., Kimball, H., and Clagett, J. Immune responsiveness of SM/J mice: cellular characteristics and genetic analysis of hyper-responsiveness to B-cell mitogens. *J. Exp. Med. 154*: 726-736, 1981. (8)

17. **Clark, E. A.**, Lake, P., and Favila-Castillo, L. Modulation of Thy-1 alloantibody responses. Donor cell-associated H-2 inhibition and augmentation without recipient Ir gene control. *J. Immunol. 127:* 2135-2140, 1981. (9)

18. **Clark, E. A.**, Sturge, J. C., and Falk, L. A. Jr. Induction of target antigens and conversion to susceptible phenotype of NK cell resistant lymphoid cell line. *Inter. J. Cancer 28*: 647-654, 1981. (5)

19. **Clark, E. A.**, Engel, D. and Windsor, N. T. Immune responsiveness of SM/J mice: hyper NK cell activity mediated by NK 1+ Qa5- cells. *J. Immunol. 127*: 2391-2395, 1981. (8)

20. Yokochi, T., Holly, R. D., and **Clark, E. A.** B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus activated B cell blasts, B lymphoid lines, and Burkitt's lymphomas. *J. Immunol. 128*: 823-827, 1982. (339)

21. Bulusu, L. K. and **Clark, E. A.** Genetic polymorphism of C3 in Papio and Macaca species. *Biochemical Genetics 20*: 859-864, 1982.

22. Nakashima, I., **Clark, E. A.**, Lake, P., Kato, N., Nagase, F., Mizoguchi, K., Isobe, K., and Saito, M. Evidence for the erythrocyte as the principal antigenic cell type

that triggers primary IgM antibody responses to H-2D alloantigens. *Transplantation 35*: 180-184, 1983. (14)

23. Ehlin-Henriksson, B., **Clark, E. A.**, Jonsson, V. and Klein, G. Studies on the B-lymphoblast antigen 1 (BB-l) on a series of Burkitt lymphoma lines differing in the expression of the EBV/C3 receptor complex. *J. Immunol. 130*: 2448-2451, 1983. (13)

24. **Clark, E. A.**, Martin, P. J., Hansen, J. A., and Ledbetter, J. A. Evolution of epitopes on human and nonhuman primate lymphocyte cell surface antigens. *Immunogenetics 18*: 599-615, 1983. (68)

25. Martin, P. J., Ledbetter, J. A., **Clark, E. A.**, Beatty, P. G., and Hansen, J. A. Epitope mapping of the human surface suppressor/cytotoxic T cell molecule Tp32. *J. Immunol. 132*: 759-765, 1984. (27)

26. Katz, F. E., Parkar, M., Stanley, K., **Clark, E. A.**, and Greaves, M. F. Chromosome mapping of cell membrane antigens expressed on activated B cells. *Europ. J. Immunol. 15*: 103-106, 1985. (24)

27. Loughran, T. P., Kadin, M. E., Starkebaum, G., Abkowitz, J. L., **Clark, E. A.**, Disteche, C., Lum, L. G., and Schlichter, S. J. Leukemia of large granular lymphocytes: Association with clonal chromosomal abnormalities and autoimmune neutropenias, thrombocytopenia, and hemolytic anemia. *Ann. Int. Med. 102*: 169-175, 1985. (195)

28. **Clark, E. A.**, Shu, G., and Ledbetter, J. A. Role of the Bp35 cell surface polypeptide in human B cell activation. *Proc. Nat. Acad. Sci. USA 82*: 1766-1770, 1985. (187)

29. Ledbetter, J. A., Tsu, T., and **Clark, E. A.** Covalent association between human thymus leukemia-like antigens and CD8(Tp32) molecules. *J. Immunol. 134*: 4250-4254, 1985. (73)

30. Wallis, W. J., Loughran, T. P., Kadin, M. E., **Clark, E. A.**, and Starkebaum, G. A. Polyarthritis and neutropenia associated with circulating large granular lymphocytes. *Ann. Int. Med. 103*: 357-362, 1985. (70)

31. Ledbetter, J. A., Rose, L. M., Spooner, C. E., Beatty, P. G., Martin, P. J., and **Clark, E. A.** Antibodies to common leukocyte antigen p220 influence human T cell proliferation by modifying IL-2 receptor expression. *J. Immunol. 135*: 1819-1825, 1985. (220)

32. Rose, L. M., Ginsberg, A. H., Rothstein, T. L., Ledbetter, J. A., and **Clark, E. A.** Selective loss of a subset of T helper cells in active multiple sclerosis *Proc. Nat. Acad. Sci. USA 82*: 7389-7393, 1985. (130)

33. Smeland, E., Godal, T., Ruud, E., Beiske, K., Funderud, S., **Clark, E. A.**, Pfeifer-Ohlsson, S., and Ohlsson, R. The specific induction of myc proto-oncogene expression in normal human B cells is not a sufficient event for acquisition of competence to proliferate. *Proc. Nat. Acad. Sci. USA 82*: 6255-6259, 1985. (126)

34. Golay, J. T., **Clark, E. A.**, and Beverley, P. C. L. The CD20 (Bp35) antigen is involved in activation of B cells from the Go to the Gl phase of the cell cycle. *J. Immunol. 135*: 3795-3801, 1985. (134)

35. **Clark, E.A.** and Ledbetter, J.A. Amplification of the immune response by agonistic antibodies. *Immunology Today 7*: 267-270, 1986. (61) ***

36. **Clark, E. A.**, Ledbetter, J. A., Holly, R. C., Dinndorf, P. A., and Shu, G. Polypeptides on human B cells associated with cell activation. *Human Immunol. 16*:100-113, 1986. (115)

37. **Clark, E. A.** and Ledbetter, J. A. Activation of human B cells mediated through two distinct cell surface differentiation antigens, Bp35 and Bp50 *Proc. Nat. Acad. Sci. USA, 83*: 4494-4498, 1986. (443)

38. Gaur, L. K., Antonelli, P., **Clark, E. A.**, and Hansen, J. A. Evolution of HLA Class 1 epitopes defined by murine monoclonal antibodies: distribution in macaques. *Human Immunol. 17*: 406-415, 1986. (8)

39. Junker, A. K., Ochs, H. D., **Clark, E. A.**, Putterman, M.L., and Wedgwood, R. J. Transient immune deficiency in patients with acute Epstein-Barr virus infection. *Clin. Immunol. Immunopath. 40*: 436-446, 1986. (19)

40. Ledbetter, J. A. and **Clark, E. A.** Surface phenotype and function of tonsillar germinal center and mantle zone B cell subsets. *Human Immunol. 15*: 30-44, 1986. (67)

41. Loughran, T. P. Jr., **Clark, E. A.**, Price, T. H., and Hammond, W. P. Acquired cyclic neutropenia is associated with increased large granular lymphocytes. *Blood 68*: 1082-1087, 1986. (45)

42. Yokochi, T., **Clark, E. A.**, and Kimura, Y. Differential expression of Epstein-Barr viral membrane antigens defined with monoclonal antibodies. *Virology 148*: 114-120, 1986. (5)

43. **Clark, E. A.** and Shu, G. Activation of human B cell proliferation through surface Bp35 (CD20) polypeptides or immunoglobulin receptors. *J. Immunol. 138*: 720-725, 1987. (71)

44. **Clark, E.A.** and Draves, K. Activation of macaque T cells and B cells with agonistic monoclonal antibodies. *Europ. J. Immunol. 17*: 1799-1805, 1987. (27)

45. Ilowite, N. T., Ochs, H. D., Rose, L. M., **Clark, E. A.**, Lindgren, K., and Wedgwood, R. J. Impaired *in vivo* and *in vitro* antibody responses to bacteriophage fX-174 in JRA. *J. Rheumatol. 14*: 957-963, 1987. (11)

46. Ledbetter, J. A., Shu, G., Gallagher, M., and **Clark, E. A.** Augmentation of normal and malignant B cell proliferation by monoclonal antibody to the B cell-specific antigen Bp50 (CDw40). *J. Immunol. 138*: 788-794, 1987. (164)

47. Loughran, T. P. Jr., Draves, K. E., Starkebaum, G. A., Kidd, P., and **Clark, E. A.** Induction of NK activity in large granular lymphocyte leukemia: activation with anti-CD3 monoclonal antibody and interleukin 2. *Blood 69*: 72-78, 1987. (48)

48. Loughran, Jr., T.P., Starkebaum, G., **Clark, E.**, Wallace, P., and Kadin, M.E. Evaluation of splenectomy in large granular lymphocytic leukemia. *Brit. J. Hematol. 67*: 135-140, 1987. (22)

49. Patarroyo, M., **Clark, E.A.**, Priesto, J., Kantor, C., and Gahmberg, C.G. Identification of a novel adhesion molecule in human leukocytes by monoclonal antibody LB-2. *FEBS Letters 210*: 127-131, 1987. (88)

50. Pezzutto, A., Dörken, B., Moldenhauer, G., and **Clark, E.A.** Amplification of human B cell activation by a monoclonal antibody to the B cell-specific antigen CD22, Bp130/140. *J. Immunol. 138*: 98-103, 1987. (103)

51. Pezzutto, A., Dörken, B., Rabinovitch, P.S., Ledbetter, J.A., Moldenhauer, G., and **Clark, E.A.** CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. *J. Immunol. 138*: 2793-2799, 1987. (138)

52. Rose, L. M., **Clark, E. A.**, Hruby, S., and Alvord, E. C. Jr. Fluctuations of T and B cell subsets in basic protein-induced experimental allergic encephalomyelitis (EAE) in longtailed macaques. *Clin. Immunol. Immunopath. 44*: 93-106, 1987. (16)

53. Rose, L.M., Alvord, Jr., E.C., Hruby, S., Jackevicius, S., Petersen, R., Warner, N.L., and **Clark, E.A.** In vivo administration of anti-CD4 monoclonal antibody prolongs survival in longtailed macaques with experimental allergic encephalomyelitis. *Clin. Immunol. Immunopath 45*: 405-423, 1987. (18)

54. Valentine, M.A., Cotner, T., Gaur, L., Torres, R., and **Clark, E.A.** Expression of the human B cell surface protein CD20: Alteration by phorbol myristate 13-acetate. *Proc. Nat. Acad. Sci. USA 84*: 8085-8089, 1987. (41)

55. Beiske, K., **Clark, E.A.**, Holte, H., Ledbetter, J.A., Smeland, E., and Godal, T. Triggering of neoplastic B cells via surface IgM and the cell surface antigens CD20 and CDw40. Responses differ from normal B cells and are restricted to certain morphologic subsets. *Int. J. Cancer 42*: 521-528, 1988. (19)

56. Benveniste, R.E., Morton, W.R., **Clark, E.A.**, Tsai, C. -C., Ochs, H.D., Ward, J.M., Kuller, L., Knott, W.B., Hill, R.W., Gale, M., and Thouless, M.E. Inoculation of baboons and macaques with SIV/Mne, a primate lentivirus closely related to human immunodeficiency virus type 2. *J. Virol. 62*: 2091-2101, 1988. (147)

57. **Clark, E.A.**, Yip, T.C., Ledbetter, J.A., Yukawa, H., Kikutani, H., Kishimoto, T. and Ng, M.H. CDw40-associated monoclonal antibodies detect two distinct molecules, which transmit progression signals to human B lymphocytes. *Europ. J. Immunol. 18*: 451-457, 1988. (20)

58. Einfeld, D.A., Brown, J.P., Valentine, M.A., **Clark, E.A.**, and Ledbetter, J.A. Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. *EMBO J. 7*: 711-717, 1988. (156)

59. Gaur, L.K., Heise, E.R., Hansen, J.A., and **Clark, E.A.** Conservation of Class I private epitopes in primate evolution. *Immunogenetics 27*: 356-362, 1988. (9)

60. Ledbetter, J.A., Rabinovitch, P.S., June, C.H., Song, C.W., **Clark, E.A.**, and Uckun, F.M. Antigen-independent regulation of cytoplasmic calcium in B cells with a 12 kDa B cell growth factor and anti-CD19. *Proc. Nat. Acad. Sci. USA 85* 1897-1901, 1988. (53)

61. Ledbetter, J.A., Tonks, N.K., Fischer, E.H., and **Clark, E.A.** CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. *Proc. Nat. Acad. Sci. USA 85*: 8628-8632, 1988. (320)

62. Makgoba, M.W., Sanders, M.E., Ginther Luce, G.E., Dustin, M.L., Springer, T.A., **Clark, E.A.**, Mannoni, P., and Shaw, S. ICAM-1: Definition by multiple antibodies of a ligand for LFA-l in B, T and myeloid cell adhesion. *Nature 331*: 86-88, 1988. (558)

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SYNGENEIC TRANSFER OF AUTOIMMUNE DIABETES FROM DIABETIC NOD MICE TO HEALTHY NEONATES

Requirement for Both L3T4$^+$ and Lyt-2$^+$ T Cells

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The nonobese diabetic (NOD) mouse was established as an inbred strain in 1980 and proposed as a model of type I diabetes mellitus (1). By 6–8 wk of age, mononuclear cells start infiltrating the periphery of pancreatic Langerhans' islets of both males and females. Progressive invasion inside the islets occurs later and is correlated with selective destruction of insulin-producing β cells and with the onset of clinically overt diabetes. Diabetes is first observed at 12 wk of age and strongly predominates in females. By 30 wk of age, ~70% of females have become diabetic, while <20% of males develop overt disease (2). Several lines of evidence suggest that diabetes in the NOD mouse is an autoimmune disease mediated by T cells: First, Thy-1,2$^+$ cells predominate in the cellular islet infiltration (3); second, neonatal thymectomy prevents the disease (4); third, NOD *nu/nu* mice do not develop diabetes (5).

Further identification of immune cells involved in the destruction of insulin-producing cells has been hindered by lack of suitable in vivo transfer models. The NOD mouse has a particular MHC haplotype due to unique I-A specificity (6) that prevents the inoculation of NOD lymphoid cells into MHC-compatible strains. Attempts to derive lines of nondiabetic mice from the original NOD nucleus have also been unsuccessful. Recently, it has been shown (7) that the transfer of spleen cells from diabetic mice into diabetes-prone NOD adults greatly promoted the onset of overt diabetes, provided that the recipients had been sublethally irradiated. In addition, adoptive transfer required recipients older than 6–8 wk who, presumably, had already begun to self-damage their pancreatic islets as inferred from histological studies (2). This latter condition limits the validity of the model to account for the whole history of β cell destruction, particularly in its initial stages.

In this study we show that diabetes can be adoptively transferred to NOD neonates by spleen cells from diabetic NOD donors. Overt diabetes, which is correlated with >90% of β cell destruction (8, 9) occurred as early as 21 d of age, at a time when pancreases of noninjected mice were still free of histological changes. The susceptibility of the recipients to the transfer was limited in time and declined after 3 wk of age. We also show that the neonatal model of transfer

A. Bendelac was supported by a fellowship from the Foundation pour la Recherche Médicale. This work was supported by funds from INSERM and Centre National de la Recherche Scientifique.

provides a sensitive in vivo assay to estimate the autoreactive potential of lymphocytes from prediabetic NOD mice. Finally, we demonstrate that diabetes can be transferred by purified T cells and that both L3T4$^+$ and Lyt-2$^+$ T cells are necessary to mediate the destruction of insulin-producing cells.

Materials and Methods

Mice. NOD mice were bred in our own facilities under specific pathogen–free conditions. The spontaneous incidence of diabetes in our colony reaches 40% by 25 wk in females, whereas overt diabetes remains rare in males. Histological examination shows that up to 92% of males and 100% of nondiabetic females present destructive insulitis by 25 wk of age, although histological alterations are usually less severe in males.

Cells. Single cell suspensions were aseptically prepared in HBSS. 4–15 spleens were pooled for each experiment. Diabetic donors were female mice used 1–3 wk after the onset of glycosuria.

Cell Fractionation. T cells were purified by filtration through nylon wool columns and subsequent panning for 45 min at 4°C over Petri dishes coated with rabbit anti–mouse Igs (Miles Scientific Div., Naperville, IL) to remove residual contaminating B cells. Depletion of Thy-1,2$^+$ cells, L3T4$^+$ cells, or Lyt-2$^+$ cells was accomplished by incubating cells at a density of 2×10^7/ml with relevant mAbs (see below) diluted in HBSS for 45 min at 4°C. The cells were then pelleted, resuspended at a density of 2×10^7/ml in rabbit complement diluted 1:30, and incubated for 30 min at 37°C. Treated cells were washed three times before injection into experimental animals. Rat IgM mAbs used for cell depletion were culture supernatants diluted 1:2 from clone 4.221 for Thy-1,2$^+$ cells (10), clone 3.155 for Lyt-2$^+$ cells (11), and clone LICR-LAU-RL172.4 for L3T4$^+$ cells (12) (with kind permission from Dr. MacDonald, Ludwig Institute for Cancer Research, Lausanne, Switzerland). For Lyt-2$^+$ cell depletion, YTS 169.4, a rat IgG2b antibody with specificity against Lyt-2$^+$ cells (Biosys, Compiègne, France), was added as an ascites fluid diluted 1:100 together with the supernatant of clone 3.155.

Monitoring of Purified Subpopulations. Contamination of purified subpopulations was monitored by membrane fluorescence analysis, using fluorescein-conjugated Fab fragments of sheep anti–mouse Igs (Biosys) for membrane Ig$^+$ cells, and fluorescein-conjugated purified ascites from clone 30-H12 for Thy-1,2$^+$ cells (13), 53-6.7 for Lyt-2$^+$ cells (13), and GK 1.5 for L3T4$^+$ cells (14). Since 30-H12 and 53-6.7 compete, respectively, with 4.221 and 3.155 for immunofluorescence staining, an additional control was included. A fluorescein-conjugated mouse anti-κ mAb specific for rat light chain (Biosys) was used to detect cells that might have escaped lysis by complement despite rat IgM-κ 4.221 or 3.155 mAbs fixation. In all depletion experiments, <1% residual cells (among $3–4 \times 10^2$ counted cells) were stained with either the corresponding antibody or the anti–rat κ light chain antibody. T cells purified after filtration through nylon wool columns and panning over Petri dishes coated with rabbit anti–mouse Igs were composed of >90% Thy-1,2$^+$ and <1% membrane Ig-bearing cells.

Neonatal Injection Protocol. Within 24 h after birth, neonates subjected to hypothermic anesthesia (4 min at −20°C) were injected into the periorbital superficial vein with 0.05 ml cell suspension at the appropriate concentration under microscopic control. For experiments designed to compare the effects of selective cell depletions or of various cell numbers, representative treatments and controls were equally distributed within each litter. Control litters were injected with spleen cells from nondiabetic NOD female mice (8–15 wk old). Moreover, to monitor any potential consequences of the neonatal manipulation on the subsequent onset of diabetes, additional controls included littermates exposed to hypothermic anesthesia and injected with HBSS.

Monitoring of Neonatally Injected Mice. Mice were tested for glycosuria three times weekly (Glukotest; Boehringer-Mannheim, Mannheim, Federal Republic of Germany) and glycosuric mice were controlled for hyperglycemia using teststrips and a quantitative colorimetric assay (Haemoglukotest and Reflolux F, Boehringer-Mannheim). Diabetic mice showed permanent fasting hyperglycemia above 3 g/liter (normal 0.88 ± 0.08), and

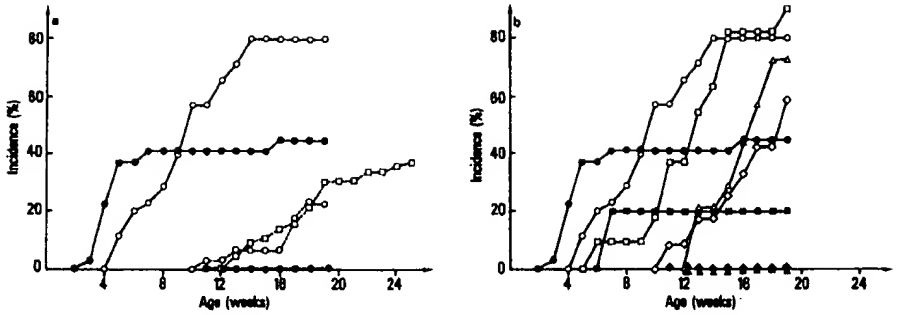

FIGURE 1. Incidence of diabetes in NOD females (*open symbols*) and males (*closed symbols*). (*a*) Broken lines, spontaneous incidence of diabetes in control groups: Neonatally HBSS-injected mice (○, $n = 31$; ●, $n = 38$), noninjected females (□, $n = 64$). Continuous lines, incidence of diabetes in mice neonatally injected with 20×10^6 cells from pooled adult diabetic female spleens (○, $n = 35$; ●, $n = 27$). (*b*) Comparison of the autoimmune potential of prediabetic NOD mice spleen cells versus that of various doses of diabetic spleen cells: Mice neonatally injected with splenocytes from diabetics, 20×10^6 cells (○, $n = 31$; ●, $n = 38$), 5×10^6 cells (□, $n = 11$; ■, $n = 10$), 1.25×10^6 cells (◇, $n = 12$; ◆, $n = 10$), or 20×10^6 spleen cells from prediabetic 8–15-wk-old females (△, $n = 14$; ▲, $n = 16$).

TABLE I

Age-related Susceptibility of Young NOD Recipients to Diabetes Transfer

Age	Successful transfers of total mice injected			
	Males		Females	
	n	%	*n*	%
1d	11/27	42	20/35	57
2d	5/10	50	6/8	75
3d	3/7	43	2/9	22
4d	2/4	50	2/4	50
3 wk	1/19	5	13/31	42
5 wk	0/29	0	1/11	9

20×10^6 spleen cells from a pool of diabetic NOD mice, injected intravenously. Successful transfers were scored up to 10 wk of life for mice injected at 1 and 3 wk and up to 15 wk of life for mice injected at 5 wk.

usually died within 2–8 wk with overt diabetic symptoms, including polyuria, polydipsia, and severe weight loss.

Histopathology. Paraffin sections (2 μM) of Bouin-fixed pancreases were stained with hematoxylin and eosin. At least 20 Langerhans islets were examined for each specimen.

Results

Adoptive Transfer of Diabetes. Diabetes can be adoptively transferred to NOD neonates by injection of adult female diabetic NOD spleen cells. As shown in Fig. 1 and Table I, up to 50% of mice injected neonatally with 20×10^6 cells became diabetic by 10 wk of age. At this age spontaneous diabetes is still not observed in the control littermates or in the other reference groups of the colony. This age was thus chosen as the upper limit for scoring successful transfers. On the other hand, >90% of the control mice of both sexes present already histological signs of insulitis (data not shown) and therefore this parameter cannot

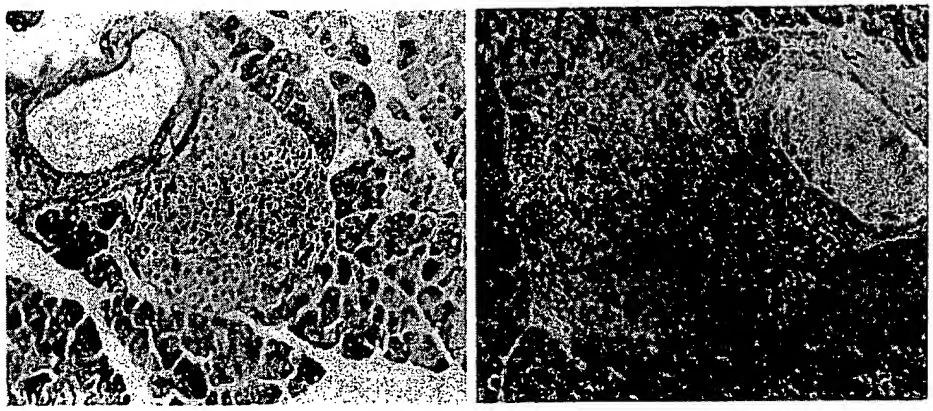

FIGURE 2. Photomicrographs of NOD mice pancreases. (*a*) Normal features in 4-wk-old NOD mice; islets of Langerhans are intact and free of mononuclear cell infiltration. (*b*) Lesions observed in a 4-wk-old male recipient neonatally injected with 20×10^6 spleen cells from diabetic adult females; severe mononuclear cell infiltration and destruction of the islet.

be used as a criterion of transfer among such animals. Some of the injected animals showed the first symptoms of diabetes as early as the third or fourth week of age. Destructive lymphocytic insulitis was observed in these mice whereas a control group of 10 males and 10 females did not present any detectable histological changes in their pancreas at 4 wk of age (Fig. 2). Importantly, diabetes could be transferred in both diabetes-prone females and diabetes-resistant males. At 10 wk, 42% of injected males and 57% of females had become diabetic. However, a marked difference between males and females occurred later. The incidence of diabetes among females increased continuously until it reached 80%, whereas it remained unchanged in males (Fig. 1).

Diabetes Transfer by Spleen Cells from Diabetics is a Dose-dependent Phenomenon. Only 19% of mice injected with 5×10^6 cells had become diabetic by 10 wk. Males and females showed similar proportions of successful transfers, but again the rate of diabetes continued to increase after 10 wk in females, whereas it remained constant in males (Fig. 1). Diabetes was not adoptively transferred with 1.25×10^6 cells. However this dose produced a significant increase in the subsequent rate of diabetes in females but not in males (Fig. 1).

Neonatal Injection of Spleen Cells from Nondiabetic NOD Mice. Groups of newborn mice were also injected with spleen cells from young nondiabetic females of 8, 10, 12, or 15 wk of age. The results obtained in these experiments were pooled to provide a substantial control group (Fig. 1). As expected, no adoptive transfer, as defined above, was observed. However a striking acceleration of the onset of diabetes was noted in these animals after 10 wk, as compared with the noninjected control groups. The incidence rate was strikingly superimposable to that observed after injection with the low dose of 1.25×10^6 spleen cells from diabetics. Interestingly, spleen cells from older nondiabetic females of 18–19 wk of age could successfully transfer the disease into newborn mice. 6 of 12 recipients became diabetic within 10 wk (data not shown).

Diabetes Transfer Depends upon the Age of the Recipients. To determine whether

TABLE II
Effect of Selective Thy-1,2$^+$ Spleen Cell Depletion on Diabetes Transfer

Injected cells	Successful transfers of total mice injected
Anti-Thy-1,2 + C′	0/6*
C′	7/11

20×10^6 pretreated viable spleen cells from a pool of six diabetic NOD mice, injected intravenously into 1-d-old recipients (two litters born on the same day).
* $p < 0.04$ (Fisher exact test).

TABLE III
Effects of Selective L3T4$^+$ and Lyt-2$^+$ T Cell Depletion on Diabetes Transfer

Exp.	Anti-L3T4 + C′	Anti-Lyt-2 + C′	C′
1	—	0/6*	6/11
2	0/6	—	1/6
3	0/3	0/4	4/4‡
4	0/6	0/6	2/7
Total			
L3T4$^+$ T cell depletion	0/15	—	7/17§
Lyt2$^+$ T cell depletion	—	0/16	12/22§

Each experiment required simultaneous intravenous injection at birth of two litters with pretreated viable spleen cells from a pool of 5–15 diabetic NOD mice. Exps. 1 and 3, 1×10^7 nylon wool–purified T cells, intact or after subset depletion; Exps. 2 and 4, 2×10^7 spleen cells, intact or after subset depletion.
* Successful transfers of total mice injected
‡ Cells injected were a mixture of 5×10^6 purified T cells depleted of L3T4$^+$ cells plus 5×10^6 purified T cells depleted of Lyt-2$^+$ cells.
§ $p < 0.02$ and $p < 0.001$, respectively, for L3T4$^+$ and Lyt-2$^+$ T cell depletion (Fisher exact test).

the transfer of diabetes was due to a particular susceptibility of newborn mice, older recipients were also injected with 20×10^6 spleen cells from diabetic mice. The results of these experiments are shown in Table I. Animals injected at the age of 1, 2, 3, and 4 d showed a similar incidence of successful transfer at 10 wk of age. Older animals were also tested as recipients. Females were still sensitive to diabetes transfer at 3 wk of age, but became refractory around 5 wk. Males, on the other hand, were already found resistant to adoptive transfer at 3 wk of age.

Lymphocyte Subsets Depletion Experiments. Table II shows that splenocytes from diabetic mice, depleted of Thy-1,2$^+$ cells no longer transfer the disease. Conversely, positively selected nylon T cells are effective in transferring diabetes (Table III, Exps. 1 and 3). Table III compares, in addition, the capacity of T cell subsets to transfer the disease. Both L3T4$^+$ cell–depleted and Lyt-2$^+$ cell–depleted splenocytes failed to transfer the disease, whereas complement-treated control spleen cells transferred diabetes. When the two T lymphocyte subsets, obtained after nylon wool passage and either anti-L3T4 or anti-Lyt-2 plus complement treatment, were mixed together, total cell number being constant

among the injected groups, successful transfer of the disease was restored (Table III, Exp. 3).

Discussion

Increasing evidence for the role of T cells in organ-specific autoimmunity has accumulated over the past few years (reviewed in reference 15). Cell-transfer experiments have provided the first direct evidence for T cell involvement in various experimental autoimmune diseases. Similar evidence has been obtained in the spontaneous autoimmune disease of the BB rat, another model of type I diabetes mellitus. The disease could be transferred from diabetic to nondiabetic BB rats provided that the injected spleen cells had been previously activated in vitro with Con A (16). However, the cells mediating the adoptive transfer have not been further characterized.

The rationale for our experiments with NOD mice was to take advantage of the delayed histological and clinical expression of the disease to develop a neonatal model of adoptive transfer that could account for the whole history of β cell autoimmune destruction. We report here several findings concerning the successful adoptive transfer of NOD diabetes to neonates, the sensitivity of the neonatal model as an in vivo assay to test the autoimmune potential of nondiabetic NOD mice, the age-related susceptibility of recipients to the transfer of the disease, and the lymphocyte subsets involved in the cellular events leading to β cell destruction.

First, type I diabetes mellitus was transferred with spleen cells from diabetic NOD mice. However, a considerable number of spleen cells were necessary to produce a significant effect. Up to 20×10^6 cells were required to destroy, in 50% of the cases, the β cells of a neonate. Several hypotheses may explain this finding: (*a*) spleen may not be the elective organ for activated autoimmune cells; attempts to transfer diabetes with lymphocytes isolated from the pancreas itself are in progress and should provide a more accurate estimation of the autoimmune potential of diabetic NOD mice lymphocytes; (*b*) β cell destruction in NOD mice, as well as in BB rats and in diabetic humans, follows a chronic course so that relatively few autoimmune cells may be needed at a given moment of the disease; and (*c*) pancreases from neonates present two distinctive features, as compared with adults, namely an increased ratio of endocrine cells and substantial regeneration potencies, as suggested by the presence of mitoses among islet cells (our histological observations) and by functional regeneration experiments (17).

Of interest was the finding that successful transfer could be achieved into both males and females, whereas only females spontaneously develop overt diabetes in our breeding colony. However, the occurrence of diabetes in the male group stopped abruptly at 7 wk after transfer, whereas the incidence in females continuously increased. Moreover, older male recipients became resistant to the transfer earlier than females, at 3 wk of age. These features are probably related to the natural resistance of males to overt diabetes. Sexual hormones, which influence the incidence of diabetes in NOD mice (18), might account for these differences. In addition, suppressor mechanisms are probably involved in the resistance of males, as suggested by the promotion of overt diabetes after cyclophosphamide treatment (19). Our results suggest that these putative mech-

anisms may influence the long-term fate of the injected cells, but do not operate in the first weeks of life.

Second, the neonatal model of transfer provides a sensitive assay to evaluate the pathogenic effect of NOD mouse lymphocytes. Although as few as 1.25×10^6 spleen cells from diabetic mice do not transfer the disease within the first 10 wk, they nevertheless increase the rate of diabetes in female recipients. This effect probably reflects the interference between adoptive transfer and spontaneous occurrence of the disease. Moreover, the finding that transfer of 20×10^6 spleen cells from 8–15-wk-old nondiabetic females can induce changes similar to those provoked by 1.25×10^6 cells from diabetics strongly suggests that the autoimmune process has already begun in prediabetic animals. Cells from older prediabetic females of 18–19 wk were able to transfer diabetes to neonate recipients with a roughly equal rate of success as spleen cells from overtly diabetic mice, indicating that the capacity of transferring the disease is not necessarily linked with the presence of overt diabetes but that it is more probably related to the duration and the severity of the insulitis as observed among 18–19-wk-old prediabetic donors (data not shown). Altogether, these data indicate that the neonatal model of transfer provides a suitable in vivo assay to explore the autoimmune potential of NOD mice at various stages of their natural history.

Third, transfer experiments with recipients of various ages suggest that the susceptibility to diabetes transfer is limited to the first weeks of life. 5-wk-old animals of both sexes appeared to be refractory to the transfer, a result which is in keeping with the work of Wicker et al. (7). However, it seems paradoxical to observe that animals of both sexes become resistant to diabetes transfer at the very age when they notoriously begin to self-damage their pancreatic islets. At variance with the adult models of transfer in NOD mice (7) and in BB rats (16), the transfer in NOD newborns does not require prior irradiation of the recipients or prior in vitro activation of the injected cells. These important features probably reflect the unique immune status of the neonate. In addition, the susceptibility of the neonate to the disease transfer provides direct evidence for the expression early in life of the pancreatic self antigen(s) involved in the autoimmune process. Therefore, the recent observations obtained in a transgenic model of antiislet autoimmunity, suggesting that the delayed expression of a (transgenic) self antigen could be responsible for the occurrence of autoimmune lesions (20), cannot be extended to the NOD mouse model.

Fourth, transfer experiments with fractionated cell subsets yield clear-cut conclusions. Both L3T4$^+$ and Lyt-2$^+$ T cells are necessary to successfully transfer diabetes to neonates, and the mixture of the two separated subsets transfers the disease. These results show that the neonatal transfer is T cell mediated and probably involves cooperation between L3T4$^+$ and Lyt-2$^+$ T cells. This dual requirement has not been observed in other models of T cell–mediated organ-specific autoimmunity in which the effector cells have been characterized, such as experimental autoimmune encephalomyelitis, adjuvant arthritis, and experimental autoimmune thyroiditis. L3T4$^+$ cells from spleen, lymph node, or cell lines and clones, presumably acting as inducer T cells, have been shown to mediate these diseases, whereas Lyt-2$^+$ cells did not seem to represent effectors in these models (15).

Identification of the phenotype of the T cells involved in the transfer does not allow, however, assigning a function to these T cell subsets in vivo. It is now admitted that the expression of L3T4 or of Lyt-2 molecules is correlated respectively with MHC class II or class I restriction of the T cell receptor rather than with the helper versus cytotoxic or suppressor functions (21). Therefore, it may only be inferred from these results that β cell destruction in the pancreas implies the presentation of autoantigen(s) in the context of class I and class II molecules at some stages of the autoimmune process. It is likely that $L3T4^+$ cells act as helper cells cooperating with activated $Lyt\text{-}2^+$ cytotoxic cells, for example by providing expansion signals such as IL-2. The absence of transfer with $L3T4^+$ cells alone would therefore indicate that the young recipients lack recruitable $Lyt\text{-}2^+$ cells. On the other hand, autoreactive $Lyt\text{-}2^+$ effector cells are probably in too limited a number in the inocula to produce the disease by themselves. Alternatively, other roles may be putatively assigned to $L3T4^+$ and $Lyt\text{-}2^+$ cells in the destruction of β cells. $L3T4^+$ cells could act as class II–restricted autoreactive cytolytic cells, as has been shown in other systems (22), and $Lyt\text{-}2^+$ cells could inhibit the secretion of insulin as indicated by in vitro experiments (23).

The identification of two cell subsets responsible for diabetes transfer does not preclude the recruitment of host lymphocytic or nonlymphocytic cells. The helper T cells might cooperate with host B lymphocytes and in turn mediate complement-dependent or antibody-dependent cell-mediated cytotoxicity. Although antiislet cell autoantibodies are detected in NOD mice (24), several lines of evidence argue against humoral effector mechanisms: (*a*) diabetes is not transferred by mothers to their offspring (our personal observation of five litters from insulin-treated diabetic mothers); (*b*) spleen cells depleted of B lymphocytes are as efficient as whole spleen cells in transferring the disease; and (*c*) B cells are underrepresented among locally infiltrating cells in the pancreas (3). Host macrophages might also be involved in the process leading to the destruction of β cells. T helper cells may secrete factors, such as macrophage-activating factor or interferon, that promote macrophage cytotoxicity. In addition, IL-1 has been shown to be selectively cytotoxic for β cells (25).

Therefore, the diabetes of the NOD mouse may result from a complex autoimmune process mediated by distinct T cell subsets. The neonatal model of transfer should provide a basis for useful and more definitive studies of the cellular events involved in the onset and regulation of autoreactivity against insulin-producing cells in type I diabetes mellitus.

Summary

We have developed a model of syngeneic adoptive transfer for type I diabetes mellitus of NOD mice. This model consists in injecting spleen cells from diabetic adult mice into newborn NOD recipients. 50% of recipients inoculated with 20×10^6 cells develop diabetes within the first 10 wk of life, at a time when none of the control littermates have yet become diabetic. The earliest successful transfers are observed at 3 wk of age, at a time when controls do not even exhibit histological changes in their pancreas. In addition we have shown that: (*a*) both males and females can be adoptively transferred, despite the fact that males rarely develop spontaneous diabetes in our colony; (*b*) diabetes transfer is a dose-

dependent phenomenon that provides an in vivo assay for comparing the autoimmune potential of spleen cells from mice at various stages of their natural history; (*c*) the susceptibility of the recipients to the transfer is limited in time and declines after 3 wk; and (*d*) both L3T4$^+$ and Lyt-2$^+$ T cell subsets are necessary for the successful transfer. The neonatal syngeneic transfer provides an effective model for studies of the cellular events involved at regulatory and effector stages of autoimmune type I diabetes.

We thank Ms. M. F. Richard for excellent technical assistance, J. Altman for managing the NOD mice colony, and C. Slama for typing the manuscript.

Received for publication 8 April 1987 and in revised form 30 June 1987.

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0022-1767/88/1418-2625$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 141, 2625–2628, No. 8, October 15, 1988
Printed in U.S.A.

ADOPTIVE T CELL TRANSFER OF AUTOIMMUNE NONOBESE DIABETIC MOUSE DIABETES DOES NOT REQUIRE RECRUITMENT OF HOST B LYMPHOCYTES[1]

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The autoimmune nonobese diabetic mouse, a model of human juvenile type I diabetes mellitus, exhibits features of both B and T cell autoreactivity against insulin-producing cells. Using the neonatal cell transfer model of the disease, which we have described previously, we have shown that B cell suppression of newborn recipients by anti-μ treatment did not affect the transfer of diabetes by means of T cells. B cell-depleted, purified T cells from diabetic adults were injected into newborns treated with either IR-52, a control rat myeloma protein, or LOMM.9, a rat anti-mouse μ-chain mAb. Both groups developed diabetes over a similar time scale. Although the pancreases in both groups showed massive infiltration by T lymphocytes, B lymphocytes, presumably recruited in the host, were present in the IR-52-treated group, whereas they were absent in the LOMM.9-treated group. Anti-μ-treated diabetic animals showed substantial B cell suppression in vivo and in vitro when compared with IR-52-treated controls. These results suggest that B cell autoreactivity is a secondary phenomenon that is unimportant during the effector phase of diabetes in nonobese diabetic mice.

Insulin-producing β cell destruction in the nonobese diabetic (NOD)[4] mouse strain results from a T cell-mediated autoimmune process. Cell-transfer experiments using diabetic adult mice as spleen cell donors and healthy neonates or irradiated adults as recipients have shown that purified T cells, but not B cells, can transfer the disease and that both $L3T4^+$ and $Lyt\text{-}2^+$ T cells are required to produce diabetes (1–2). However, these findings did not exclude the possibility that some of these T cells might recruit host non-T cells, which in turn could participate in the destruction of insulin-producing cells. Macrophages are potential effectors of this process, since they are present in the islet infiltrate and may produce IL-1, a monokine with a selective cytostatic action on β cells in vitro (3). B lymphocytes are also present in the pancreases of diabetic mice (4), and some investigators have reported a humoral response against islet cell Ag such as insulin and other not yet fully defined cytoplasmic or cell surface Ag (5).

To investigate the role of B cells as putative effectors of the autoimmune destruction of insulin-producing β cells in the NOD mouse, we have injected highly B cell-depleted, purified T cells from adult diabetic donors into newborns that were actively B cell suppressed by a regimen of chronic injections of a rat anti-mouse μ chain mAb. Our results show that the T cell-mediated transfer of diabetes is not affected by B cell suppression and, therefore, suggest that B cells are not required at the effector level during the autoimmune process leading to diabetes mellitus in NOD mice.

MATERIALS AND METHODS

Mice

NOD mice were bred in our own facilities under specific pathogen-free conditions. The spontaneous incidence of diabetes in our colony reaches 40% by 25 wk in females, whereas diabetes remains rare in males.

Cells

Single cell suspensions were aseptically prepared in HBSS. Spleens from 15 spontaneously diabetic females were pooled for cell transfer experiments.

T Cell Purification and Monitoring of Contaminating B Cells

The protocol has been detailed previously (1). Briefly, T cells were purified by filtration through nylon wool columns and subsequent panning for 45 min at 4°C over petri dishes coated with rabbit anti-mouse Ig (Miles Scientific Division, Naperville, IL) to remove residual B cells. Contamination of the purified T cell subset was monitored by membrane fluorescence analysis using fluorescein-conjugated Fab fragments of sheep anti-mouse Ig (Biosys, Compiègne, France) for membrane Ig^+ cells, and fluorescein-conjugated purified antibodies from clone 30H12 for $Thy\text{-}1\text{-}2^+$ cells. Purified cells were composed of >95% $Thy\text{-}1\text{-}2^+$ cells, whereas <0.5% residual B cells were present (among 8×10^2 counted cells).

Protocol for B Cell Suppression In Vivo

B cell suppression was induced using i.p. injections of a single purified rat anti-mouse μ-chain mAb, LOMM.9 (6) at a dilution of 4 mg/ml in sterile PBS. Control mice were injected with IR-52, a rat myeloma protein of the same isotype, IgG2a-kappa, as LOMM.9, at the same dilution. Treatment was started within the first hours of life by the injection of 0.1 ml of the mAb. Additional injections of 0.1 ml were given at day 2 and day 4, then treatment was maintained by two weekly injections of 0.2 ml of the mAb until the onset of diabetes.

Received for publication April 25, 1988.
Accepted for publication July 13, 1988.
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[1] This work was supported by funds from INSERM and CNRS.
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[4] Abbreviations used in this paper: NOD, nonobese diabetic.

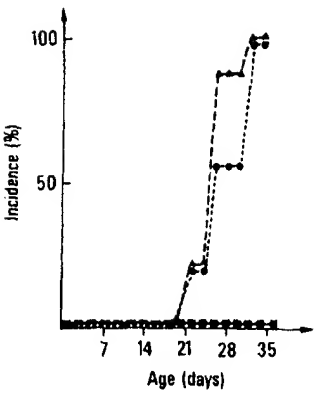

Figure 1. Incidence of diabetes in B cell suppressed NOD mice. Mice neonatally injected with 20 × 10^6 purified T cells from diabetic donors were B cell-suppressed with injections of the anti-μ LOMM.9 mAb (▲, n = 9) as described in *Materials and Methods*, or treated with the control myeloma protein IR-52 (●, n = 9). Control mice (■, n = 9) were treated with LOMM.9 without T cell transfer.

Neonatal Cell Transfer

Neonates from two litters born on the same day were injected i.v. with 20 × 10^6 purified T cells in 0.05 ml HBSS. Nine were treated with LOMM.9 and nine with IR-52. An additional control included two litters injected with LOMM.9 alone.

Monitoring the Onset of Diabetes in Neonatally Injected Mice

Mice were tested for glycosuria four to five times a week, and glycosuric mice were monitored for hyperglycemia as previously described (1). Diabetic mice showed fasting hyperglycemia above 3 g/L (normal levels 0.88 ± 0.08 g/L).

Monitoring of B Cell Suppression

Mice were sacrificed 24 to 72 h after the onset of diabetes and assessed for B cell suppression as follows: 1) B cells in spleen cell suspensions were detected by membrane Ig staining using fluorescein-conjugated Fab fragments of sheep anti-mouse Ig (Biosys). As an additional control, anti-Ia labeling with a biotinylated mAb, 10.3.6 (7), and fluorescein-conjugated avidin, was performed in some animals: 2) Ig-secreting spleen cells were detected by the protein A plaque assay: cells were cultured at a concentration of 2 × 10^5/200 μl in flat-bottomed microculture plates in RPMI 1640 medium supplemented with antibiotics, glutamine, 2-ME (5 × 10^{-5} M) and 10% FCS. Triplicate cultures were set up in the presence of LPS at 25 μg/ml (Difco Laboratories, Inc., Detroit, MI) or without this mitogen. After a 4-day stimulation period, cells were recovered and tested with the staphylococcal protein A plaque assay using a rabbit anti-total mouse Ig serum as previously described (8); 3) levels of circulating IgG and IgM were determined using a solid phase ELISA assay with isotype-specific antibodies for coating and development and purified IgG or IgM as standards (Miles Scientific Div.). No significant cross-reaction was found with the rat mAb LOMM-9 and IR-52 injected in vivo.

Immunohistochemical Examination of Mouse Pancreases

Frozen sections (4-μm) of pancreases from two diabetic mice in the LOMM-9 injected group and from two diabetics in the IR-52 injected group were labeled with biotinylated F(ab')$_2$ fragments of sheep anti-mouse Ig antibodies (Amersham Corp., Les Ulis, France) and with a biotinylated anti-Thy-1.2$^+$ mAb, 30H12 (9), followed by the standard avidin-biotin-peroxidase complex method as described elsewhere (10). A total of 15 to 30 islets were examined for each specimen.

RESULTS

As shown in Figure 1, diabetes was successfully induced in all of the mice treated with anti-μmAb, after the transfer of 20 × 10^6 purified T cells from diabetic NOD donors. Furthermore, the onset of diabetes was not delayed in that group as compared with control IR-52 treated mice. Mice receiving anti-μ alone without T cell transfer did not develop the disease during the course of the study.

Table I shows that a substantial B cell suppression was achieved during the first weeks of life in those animals receiving a regimen of injections of a single rat anti-mouse μ-chain mAb, as previously reported (11). Moreover, B cell suppression was not affected by the neonatal transfer of the B cell-depleted, purified T cells. The reality of B cell depletion was also assessed in some mice using an additional labeling with the 10.3.6 anti-Ia mAb: less than 8% positive cells—presumably monocytes—were

TABLE 1

B cell suppression in anti-μ (LOMM.9)-treated NOD mice[a]

Age	Treatment											
	LOMM-9				LOMM.9 + T Cell Transfer				IR-52 + T Cell Transfer			
	% Ig$^+$ cells[b]	PFC-protein A[c]	IgM[d]	IgG[d]	% Ig$^+$ cells	PFC-protein A	IgM	IgG	% Ig$^+$ cells	PFC-protein A	IgM	IgG
17 days	<0.5		<.01	760								
	<0.5		<0.1	1010								
	<0.5		<0.1	660								
22 days	1	<100	<0.1	430	1	<100	<0.1	650	31	7100	1040	1500
	1	<100	<0.1	500	1	<100	<0.1	445	32	4800	1690	890
	2	<100	<0.1	470								
26 days	2		<0.1	680	1	<100	<0.1	1200	41	5400	690	3560
	2		<0.1	450	2	200	<0.1	1400	36	8800	1500	2430
	4		<0.1	390	2	300	<0.1	530	40	7600	1810	4240
					3	200	<0.1	440				
					6	400	<0.1	490				
					8	300	<0.1	630				
32 days					12		<0.1	780	42			
									45			
									46			
									43			

[a] NOD mice were injected i.v. at birth with 20 × 10^6 purified T cells from a pool of diabetic NOD mice and received chronic i.p. injections of LOMM.9, an anti-μ rat mAb, or of IR-52, a control myeloma protein, as described in *Materials and Methods*. To assess B cell suppression, mice were killed 24 to 72 h after the onset of diabetes. As a control, mice injected with anti-μ alone were simultaneously killed.

[b] Spleen B cells positive for membrane Ig immunofluorescence staining (percentage among 8 × 10^2 counted cells).

[c] Number of Ig secreting cells in the protein A plaque assay for 10^6 spleen cells, after 4 days of culture with LPS, 25 μg/ml. Background level for non-mitogen-stimulated cultures is <100. PFC, plaque-forming cells.

[d] Serum level of IgM and IgG, as determined using a solid phase ELISA assay (μg/ml).

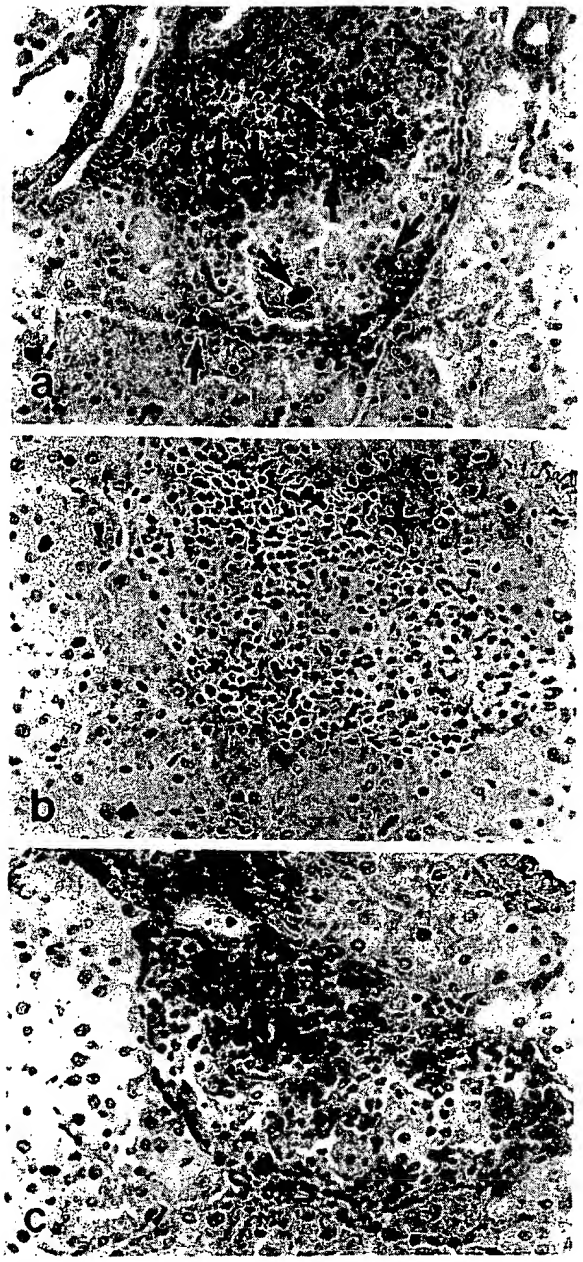

Figure 2. Immunoperoxidase staining of Ig$^+$ and Thy-1.2$^+$ cells in the islets of Langerhans infiltrate of diabetic NOD mice. (*a*) scattered B cells are present around and inside the islets of control mice (*arrows*). (*b*) and (*c*) serial sections in B cell suppressed mice: (*b*) B lymphocytes are missing; (*c*) most inflammatory cells are Thy-1.2$^+$ lymphocytes.

detected in deeply B cell-depleted mice, as compared to 30 to 40% in controls. Partial escape from suppression was observed after 4 wk of anti-μ treatment as evidenced by the reappearance of detectable membrane Ig$^+$ spleen cells, whereas functional suppression of Ig production tested in vitro by the protein A plaque assay and in vivo by the levels of circulating IgM lasted longer. IgG levels in anti-μ-treated young NOD mice were only slightly reduced, suggesting that they were residual IgG of maternal origin (11). Finally, immunolabeling experiments revealed that anti-μ treatment was able to delete B lymphocytes from the pancreatic infiltrate without affecting the expression of the autoimmune disease. Furthermore, similar patterns of lymphoid infiltration and of islet destruction were observed in both anti-μ-treated and control diabetic recipients of the T cell transfer (Fig. 2).

DISCUSSION

While the detection of autoantibodies has provided the historical basis for the autoimmune origin of various disorders, converging arguments point now to the concurrent involvement of T cell-mediated immunity (12). Type I diabetes mellitus in humans, as in the animal models of the BB rat and the NOD mouse, presents features of both B and T cell autoreactivity. Antibodies directed against islet cell Ag can be detected in the sera of diabetics (5, 13–14); on the other hand, T cells predominate over B cells in the lymphocytic infiltrate in the islets of Langerhans (4, 15–16), and in NOD mice the disease transfer requires both L3T4$^+$ and Lyt-2$^+$ T cells (1–2). To investigate whether B cell infiltration of the pancreas and autoantibodies production are necessary for disease expression or whether they mainly reflect secondary events, adoptive T cell transfer of diabetes was performed into B cell-suppressed or control, newborn recipients.

The results show that host B cells are recruited in situ in the pancreases of control recipients injected with B cell-depleted, purified T cells. However, the recruitment of B cells either in situ or in the periphery is definitely not important for disease expression, since B cell-suppressed recipients exhibited the same susceptibility to diabetes transfer as controls. These conclusions are in keeping with our previous observation that purified T cells are as efficient as whole spleen cells in transferring the disease (1). Although residual IgG from maternal origin can be detected in diabetic anti-μ-treated mice, it is unlikely that they participate in the pathologic process for two reasons. First, passive transplacental transfer of diabetes is not observed in litters from diabetic mothers (1), and second, the injection of uncommitted spleen cells—from nondiabetic mice—as a source of antibody-dependent-cell-mediated-cytotoxicity effector cells is unable to produce the disease (1). Autoimmune damage probably induces secondary events that are not directly relevant to pathogenesis. Our histologic data suggest that not all the cell subsets infiltrating the islets of Langerhans are required for the autoimmune destruction of β cells. Similar conclusions have been reached in recent studies on experimental allergic encephalomyelitis showing that most of the infiltrating cells, especially macrophages, are not necessary for the production of disease (17).

Thus, B and T cell autoreactivity can be dissociated in the NOD mouse. Whether B cell autoreactivity is restricted to pancreatic Ag and whether it is linked to the genetics of the T cell-mediated autoimmune disease of the pancreas (18) is currently under investigation in our laboratory. The results presented in this study suggest that B cells do not play an important role during the effector phase of the disease. The destruction of insulin-producing cells in diabetes of the NOD strain appears therefore to involve a restricted number of immune cell subsets, namely L3T4$^+$ and Lyt-2$^+$ T cells, while the putative role of macrophages needs further investigation.

Acknowledgments. We would like to thank B. Legrand and M. F. Richard for excellent technical assistance, J.

Altman for managing the NOD mice colony, and C. Slama for typing the manuscript.

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Interaction Between CD40 and Its Ligand gp39 in the Development of Murine Lupus Nephritis[1]

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We investigated the role of gp39-CD40 interaction in the development of glomerulonephritis in lupus mice. In contrast to normal mice, lupus mice had much higher percentages of intensely gp39$^+$ T cells in their spleens even at the preautoimmune age of 1 mo, and the further increase in gp39 expression by anti-CD3 Ab stimulation was markedly greater in lupus T cells. The pathogenic autoantibody-inducing ability of Th clones and splenic Th cells from lupus mice could be blocked in vitro by anti-gp39 Ab. Acceleration of lupus nephritis by the transfer of pathogenic autoantibody-inducing Th clones in vivo could also be completely blocked by anti-gp39 Ab. Surprisingly, a brief treatment of lupus mice with anti-gp39 Ab had a sustained beneficial effect on their spontaneous disease long after the Ab had been cleared from their systems. Only three injections of anti-gp39 Ab given to prenephritic lupus mice at 3 mo of age markedly delayed and reduced the incidence of lupus nephritis up to 12 mo of age by which time almost all the control mice had developed severe glomerulonephritis. Remarkably, pathogenic Th cells were left intact in these anti-gp39-treated mice but their B cells could not produce pathogenic autoantibodies even 9 mo after the therapy. Our studies suggest that blocking the interaction between gp39 on pathogenic Th cells and CD40 on lupus B cells at a crucial window of time delays the expansion autoimmune memory B cells resulting in long-term therapeutic benefits. *The Journal of Immunology,* 1995, 154: 1470–1480.

In the spontaneously arising systemic autoimmune disease, SLE,[3] the pathogenic autoantibody-producing B cells are driven by certain Th cells (1, 2). These autoimmune Th cells have been cloned from mice and patients with lupus nephritis (3–7). In the (SWR × NZB)F_1 (SNF_1) mice that develop fatal lupus nephritis, these pathogenic autoantibody-inducing Th clones bear a recurrent motif of anionic residues in the CDR3 loops of their TCRs suggesting that they are specific for autoantigens with cationic residues. Indeed, about 50% of the Th clones respond to Ags that are derived from nucleosomes and presented by MHC class II molecules (8–10). Because these autoimmune Th clones selectively help pathogenic anti-DNA autoantibody-producing B cells, those B cells probably process and present the charged nucleosomal Ags to these Th cells (9). These observations and the fact that pathogenic autoantibodies have the characteristics of an Ag-driven response such as IgG class switch, clonal expansion, and somatic mutation (reviewed in Ref. 11) all indicate that a cognate interaction between the pathogenic Th and B cells is occurring in SLE (3, 8, 9, 12, 13). However, because polyclonal B cell hyperactivity is an early feature of SLE preceding the pathogenic autoimmune response (14, 15), it has been implied in some studies that the hyperactive B cells could produce autoantibodies as mere bystanders after receiving lymphokines produced by any activated T cell (16). In this situation a cognate interaction would not be necessary. During cognate, contact-dependent help for Ab production, gp39 is transiently expressed on activated Th cells, and it binds to CD40 on the Ag-specific B cells transducing a second signal that is essential for B cell growth and differentiation (17–19). The CD40-transduced signal also prevents apoptosis of germinal center B cells that have encountered Ag (20). To establish the

Received for publication July 28, 1994. Accepted for publication October 22, 1994.

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[1] This work was supported by National Institutes of Health Grants CA31789 and AR39157 to S.K.D., an Arthritis Foundation Fellowship to C.M., and TALENT-stipend from Netherlands Organization of Scientific Rersearch to J.D.L.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; SNF_1, (SWR × NZB)F_1 mice; CDR, complementarity determining region; SAS, saturated ammonium sulfate; Hlg, hamster Ig; MFI, mean fluorescence intensity; S-PE, streptavidin-phycoerythrin.

0022-1767/95/$02.00

importance of a contact-dependent, cognate interaction between the pathogenic Th and B cells of lupus, we tested the effect of an Ab to the CD40-ligand (gp39 or CD40-L) in blocking the pathogenic autoimmune response.

Materials and Methods

Mice

BALB/c, NZB, and SWR mice were from The Jackson Laboratory (Bar Harbor, ME). SNF_1 hybrids were bred at Northwestern University Animal Facility, Chicago, IL. Female mice were used for the studies.

Abs

The following mAbs: TIB120/M5 broadly reactive against $I\text{-}A^{b,d,q}$ and $I\text{-}E^{d,k}$; anti-$I\text{-}A^d$ (HB3/MKD6), anti-J11d (TIB183), anti-Thy-1.2 (TIB99), anti-CD8 (TIB211), and anti-CD3 (145–2C11), were obtained from the American Type Culture Collection (ATCC, Rockville, MD. References in catalog), concentrated 10 times by 47% saturated ammonium sulfate (SAS) cutting of hybridoma culture supernatants, and dialyzed before use. MR1, hamster anti-murine gp39 mAb (21), was purified by DEAE HPLC from ascites fluid. Hamster Ig (HIg), used as a control Ab, was purified similarly from hamster serum (Accurate Chemical and Scientific Corp., Westbury, NY). For FACS analysis, these Abs were biotinylated using standard procedures. FITC-conjugated mAb to CD4 and streptavidin-PE (S-PE) were purchased from Life Technologies (Bethesda, MD).

T cell clones

The Th lines and hybridomas used in this study were derived from nephritic SNF_1 mice, subcloned, and maintained as previously described (3, 4, 8). These Th clones induce pathogenic autoantibodies when cocultured with SNF_1 B cells and they can also induce lupus nephritis in young preautoimmune mice, upon transfer in vivo (8).

Isolation of splenic T cells

Whole spleen cells from which E had been removed by hypotonic lysis were depleted of B cells by one cycle of panning (4). The nonadherent cells (whole T cells) were further purified for $CD4^+$ T cells by C-mediated lysis as described (1, 9). Spleen cells were suspended at 20×10^6/ml in serum-free medium containing a cocktail of Abs: anti-CD8 (1/4), anti-Ia (1/4), and anti-J11d (1/10) for 30 min on ice. Ab treatment was followed by incubation with a mixture of rabbit (1/20) and guinea-pig (1/10) C (Pel Freeze Biologicals, Rogers, AR) at 37°C for 45 min. In some experiments, the functional status of splenic T cells was verified by their proliferative response to anti-CD3 or mononucleosome-pulsed APCs, as previously described (9).

Activation of T cells by anti-CD3

Twenty-four-well Costar plates were precoated with 10 μg/ml anti-CD3 (1 ml/well) for 3 h at 37°C and washed twice with PBS. Splenic T cells or Th clones were cultured (1×10^6/ml) in these anti-CD3 precoated wells for 6 to 8 h before flow cytometric analysis.

Preparation of splenic B cells

Whole spleen cells depleted of E were incubated at 20×10^6/ml in serum-free medium containing anti-Thy-1.2 (1:4) for 30 min on ice, followed by rabbit C (1/5) at 37°C for 45 min. This B cell-enriched suspension was cocultured at 2.5×10^6/well with T cells in 2 ml final volume in 24-well Costar plates for helper assays.

Flow cytometric analysis

Resting and activated T cells were harvested and washed in ice-cold PBS/5% FCS/0.05% sodium azide (FACS buffer). FcR were first blocked by preincubating cells in FACS buffer containing 10% normal rabbit serum. Cells were labeled with 10 μg/ml biotinylated MR1 or control HIg-biotin for 40 min in ice. These cells were then washed and counterstained with S-PE at 10 μg/ml. Two color staining with two different mAbs was done as described (2, 4). Stained cells were analyzed using a Becton Dickinson FACScan flow cytometer. Cells were gated according to their forward- and side-scatter characteristics. At least 10,000 viable cells were analyzed for the definition of percent positive cells and mean fluorescence intensities (MFI). Cells with MFI between 10 and 100 were defined as "dull-positives," whereas cells with MFI >100 were deemed "bright-positives" for MR1 staining. Data were analyzed using Lysis II software (Becton Dickinson).

Immunohistochemistry

Cryosections were made of spleens frozen in liquid nitrogen, kept overnight in humidified atmosphere, air dried, and fixed in fresh acetone/0.02% hydrogen peroxide for 10 min. After air drying, slides were rehydrated with PBS and incubated for 16 h at 4°C with biotinylated MR1, washed with PBS, incubated with avidin-HRP for 1 h at room temperature, washed, and stained for 10 min with AEC, producing a red precipitate as previously described (22). Alternatively, MR1 was used, followed by RG7-biotin (an anti-rat IgG that cross-reacts with HIg). Slides were washed and sections were counterstained with hematoxylin and embedded with glycerol gelatin.

Helper assays

The pathogenic clone L-3A (1×10^6/well) or SNF_1 splenic T cells (2.5×10^6/well) were cocultured with SNF_1 splenic B cells (2.5×10^6/well) in 24-well Costar plates for 7 days, as previously described (1, 3). On day 7, culture supernatants were harvested, freeze-thawed, and assayed by ELISA for Abs against ssDNA, dsDNA, histones, and histone-DNA complex. For these ELISAs, the supernatants were purified by 47% SAS cutting, dialyzed, and used as described below. For blocking studies, MR1 was added to the cocultures at 10 μg/ml, throughout the 7-day assay.

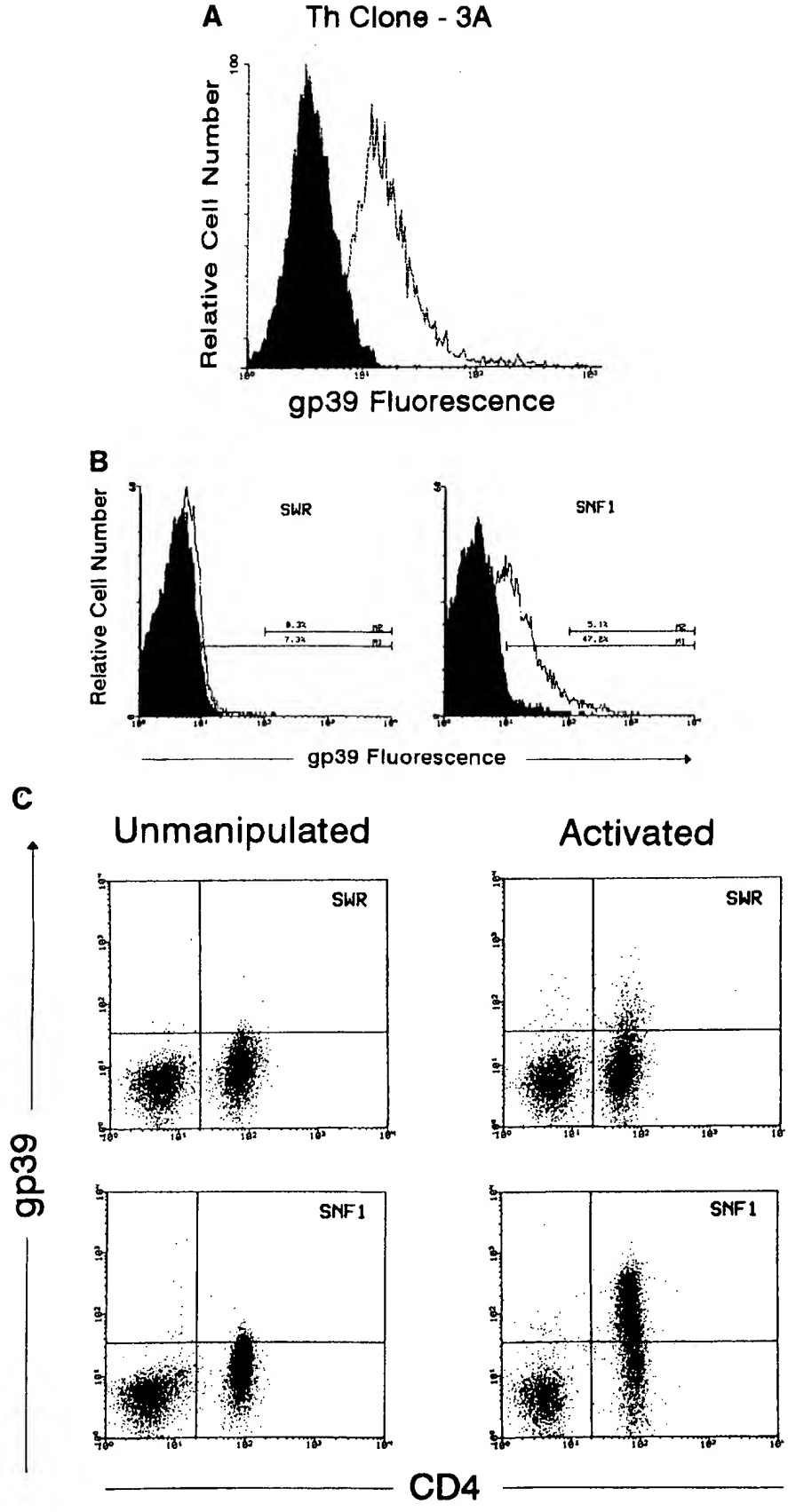
ELISA

Total IgM and IgG, and IgG class autoantibodies to ssDNA, dsDNA, histones, and histone-DNA complex were measured as described (1, 3, 9). Sera were diluted 1/100, and heat inactivated at 56°C before use. Culture supernatants were concentrated four times by SAS cutting before being applied to the ELISA plates. Standard curves for each assay were obtained with known quantities of anti-DNA mAbs 564 and 205 as described (1, 3, 9). For IgG autoantibodies to ssDNA, histones, and histone-DNA complex, 1 U/ml was considered to be equivalent to the activity of 1 μg/ml of mAb 564, which bound to all three Ags; for IgG anti-dsDNA, 1 U/ml was equivalent to the binding of 1 μg/ml of the mAb 205.

Anti-gp39 treatment

Long-term study. Three-month-old preautoimmune SNF_1 mice (10 per study group) were injected with sterile, HPLC-purified anti-gp39 (MR1) or HIg (as a control Ab that was preabsorbed with normal mouse spleen cells to remove nonspecific xenoreactivity) i.p. (250 μg/mouse) every other day for 3 days, i.e., on days 0, 2, and 4. The mice were monitored weekly for proteinuria by albustix (Miles Laboratories, Elkhart, IN (23)), and killed when they developed persistent proteinuria (defined as two consecutive weekly recordings of 300 mg/dl or greater). Sera were collected at 7 mo of age and also upon killing for the determination of IgG autoantibody levels by ELISA and of blood urea nitrogen by azostix (Miles). Sections from the kidneys were stained for detection and grading of glomerulonephritis by light microscopy, and immunofluorescence of frozen sections to detect the degree of IgG immune complex deposits was done as described (8, 9, 23). Dr. Yashpal Kanwar (Department of Pathology, Northwestern University Medical School, Chicago, IL) also evaluated the same kidney sections in a blinded fashion. Upon killing of the mice, spleens were used for helper and other functional assays, and for the detection of $gp39^+$ T cells by flow cytometric analysis or by immunohistochemistry.

Lupus acceleration study. In an effort to accelerate the onset of lupus nephritis (8), a second batch (six per group) of 3-mo-old SNF_1 mice were injected i.v. with the pathogenic Th clone L-3A (1×10^7 cells/mouse) on day 1. In addition, on days 0, 7, 14, 21, and 28, an experimental group of


A
Th Clone - 3A
Relative Cell Number
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B
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SNF1
Relative Cell Number
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SNF1
SNF1
gp39
CD4

Table I. *gp 39 expression by splenic T cells*

Mice	n	% of gp39$^+$ Cells Among CD4$^+$ T Cells in Spleen: Unmanipulated ex vivo, Dull + Bright[a]	Unmanipulated ex vivo, Bright[b]	Activated by anti-CD3[c]
		Mean ± S.E.M.		
SWR 1 mo	7	2.01 ± 0.78	0.45 ± 0.18	15.14 ± 4.96
SNF_1 1 mo	6	18.49 ± 5.73	2.48 ± 0.55	47.88 ± 8.64
SNF_1 6 mo	6	25.22 ± 5.19	4.01 ± 0.74	53.05 ± 4.85

[a] Dull + Bright = All gp39$^+$ cells. Corresponds to M1 in Figure 1*B* (single color immunofluorescence). The mean fluorescence intensities (MFI) were 58.71 ± 8.27 for SWR, 56.67 ± 4.80 for SNF_1 1 mo, and 51.79 ± 3.74 for SNF_1 6 mo.

[b] Bright = Strongly positive for gp39 expression, corresponding to M2 in Figure 1*B*. The MFI were 369.19 ± 45.08 for SWR, 612.30 ± 171.61 for SNF_1 1 mo, and 511.85 ± 100.74 for SNF_1 6 mo.

[c] Values represent all gp39 positive cells (bright + dull) after anti-CD3 activation in vitro.

mice received 250 μg MR1 i.p., whereas the control group of mice received 250 μg HIg i.p. These mice were monitored for the development of lupus nephritis, as described above.

Results

Expression of gp39 by pathogenic autoantibody-inducing Th clones and splenic T cells of lupus mice

As described previously, a minor proportion (12%) of 268 autoreactive T cell clones derived from the T cells that are spontaneously activated in vivo in SNF_1 mice with lupus nephritis have the ability to induce pathogenic anti-DNA autoantibodies in vitro (3, 4, 8). About 50% of these pathogenic autoantibody-inducing Th clones are specific for nucleosomes, and representative clones could rapidly induce lupus nephritis when transferred in vivo (8, 9). Here, we tested nine of the pathogenic, CD4$^+$ Th clones from SNF_1 mice and found that all of them expressed high levels of gp39 (56 to 84% positive) upon stimulation by anti-CD3, similar to anti-CD3-stimulated T cell clones from normal mice (24). A representative pathogenic Th clone, 3A, is shown in Figure 1.

Table I and Figure 1 show that in contrast to the T cells of normal SWR parents the proportion of freshly isolated, CD4$^+$ T cells from the lupus-prone SNF_1 mice that spontaneously expressed gp39 was significantly higher both at preautoimmune stage of 1 mo of age ($p < 0.05$, two-tailed *t*-test) and also at 6 mo with overt disease ($p < 0.01$). The subpopulation of these T cells that stained brightly for gp39 expression was also markedly increased in the SNF_1 at 1 mo ($p < 0.02$) and at 6 mo ($p < 0.01$) as compared with the SWR T cells. Upon further stimulation in vitro by anti-CD3, the increase in the percentage of gp39$^+$ T cells was much higher in the SNF_1 lupus mice than in the normal SWR mice ($p < 0.05$ at 1 mo and $p < 0.001$ at 6 mo). Although there was an apparent increase in gp39 expression by T cells of SNF_1 mice at 6 mo as compared with the SNF_1 mice at 1 mo of age, this increase was not statistically significant (Table I). The proportion of gp39-expressing T cells in SWR mice was similar at 1 mo and 6 mo (not shown) and the values were similar to those in other normal strains such as, BALB/c, C57BL/6 and (SWR × BALB/c)F_1 (results not shown) (24).

Immunohistochemical staining of spleen sections for gp39 expression supported the flow cytometry results (Fig. 2). Spleens from six 1-mo-old and seven 6-mo-old SNF_1 mice were examined and they contained higher numbers ($p < 0.01$) of gp39$^+$ T cells (ranging from 25 to 300/section; mean ± SEM = 79.1 ± 19.9) than the spleens of four SWR mice examined (from 0 to 10/section; mean ± SEM = 5.0 ± 2.2).

Ab to gp39 blocks T cell help for pathogenic autoantibody production in vitro

The production of nephritogenic, IgG class autoantibodies to DNA and nucleosomes (histone/DNA complexes) by lupus B cells can be induced in vitro by coculturing with CD4$^+$ Th cells from the spleens of older SNF_1 mice (1) or by the pathogenic Th clones derived from the SNF_1 mice (3, 4, 8). The pathogenic Th clones augmented the production of IgG autoantibodies when cultured with syngeneic B cells in the presence of the control HIg, but this help was markedly inhibited in the presence of the anti-gp39 mAb, MR1, in the cocultures ($p < 0.02$ to $p < 0.001$ for the different autoantibodies). Results with a representative Th clone 3A is shown in Table II. Similarly, the autoantibody-inducing help of freshly isolated CD4$^+$ T cells from older prenephritic SNF_1 mice was markedly blocked by anti-gp39 (Table II). The autoantibody-inducing help of the Th cells in the presence of the control HIg

FIGURE 1. Representative flow cytometry analysis of gp39 expression. *A*, pathogenic autoantibody-inducing Th clone derived from a nephritic SNF_1 mouse before (solid) and 7 h after activation with anti-CD3 Ab. *B*, freshly prepared CD4$^+$ T cells from the spleens of SWR (1-mo-old) and SNF_1 (1-mo) mice. Background staining (solid) and gp39 staining (open histograms) are shown within the range marked by M1, for all positive cells (dull + bright) and the range M2, for only the brightly staining cells. The percent values represent all cells stained by anti-gp39 (MR1) that fall within M1 or M2, without subtraction of background staining values (net values after background subtraction are shown in Table I). *C*, two color immunoflurescence analysis of whole (unfractionated) T cell population from the spleens of SWR (1 mo) and SNF_1 (1-mo) mice. "Unmanipulated" means analyzed fresh ex vivo. "Activated" means after stimulation with anti-CD3 in vitro. gp39 (red) fluorescence and CD4 (green) fluorescence are shown on *y*- and *x*-axes, respectively. Based on control staining, the dot-plots were divided into four quadrants: unstained cells (*lower left*); cells that stained with both Abs (*upper right*), and cells that stained with only one of the two Abs (*upper left* and *lower right*).

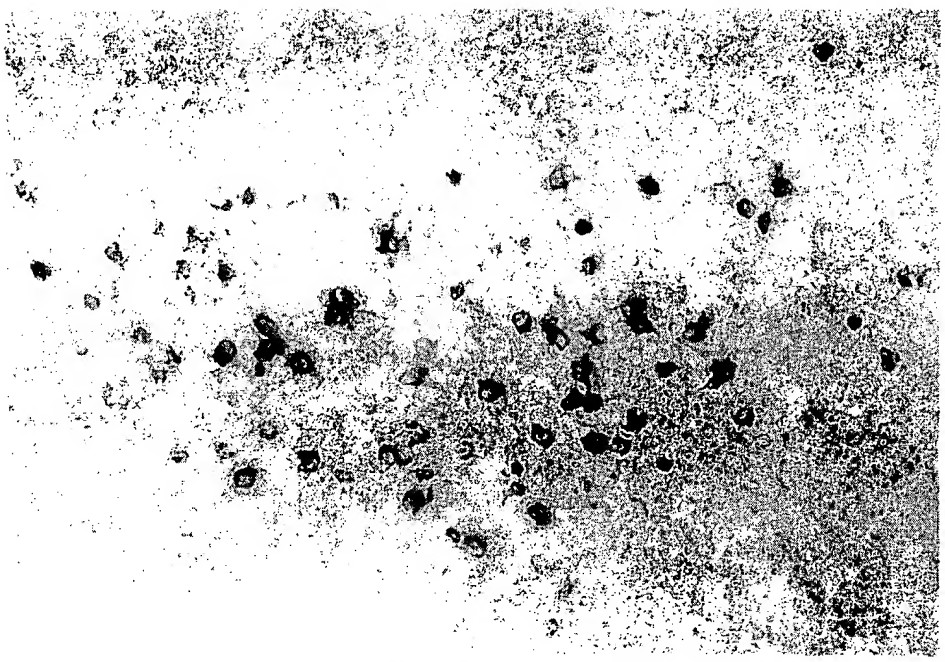

FIGURE 2. Immunohistochemical detection of gp39-expression in the spleen of lupus mice. gp39-positive T cells were detected in frozen spleen sections by staining with anti-gp39 Ab (MR1). A group of gp-39 expressing cells in the spleen of a 6-mo-old female SNF_1 mouse is shown. Similar results were obtained with spleens from 1-mo-old SNF_1 mice.

Table II. *Blocking of autoantibody-inducing help by anti-gp39 (MR1) Ab*

Culture Conditions[a]	IgG Autoantibody Production in Culture U/ml (mean ± SEM)			
	Anti-ssDNA	Anti-dsDNA	Anti-histone	Anti-histone/DNA
Help with Th clone[b]:				
B cells alone	0.61 ± 0.03	1.49 ± 0.07	0.29 ± 0.18	1.01 ± 0.15
B + clone 3A + Hlg	7.91 ± 0.07	6.16 ± 0.52	14.41 ± 1.14	7.61 ± 0.87
B + clone 3A + MR1	3.78 ± 0.53	2.53 ± 0.10	1.76 ± 0.49	0.78 ± 0.11
% inhibition by MR1	52	59	88	90
Help with splenic T cells[c]:				
B cells alone	0.19 ± 0.02	0.24 ± 0.01	3.78 ± 0.04	0.32 ± 0.05
B + $CD4^+$ T + Hlg	0.45 ± 0.03	2.97 ± 0.35	13.45 ± 1.41	3.49 ± 0.46
B + $CD4^+$ T + MR1	0.16 ± 0.02	0.28 ± 0.01	3.12 ± 0.20	0.44 ± 0.05
% inhibition by MR1	65	91	77	87

[a] A representative of three experiments is shown for each set. MR1 = anti-gp39 mAb. Hlg = control hamster Ig.
[b] In this experiment, the pathogenic autoantibody-inducing Th clone 3A derived from SNF_1 was used to augment autoantibody production by splenic B cells from a 4.5-mo-old SNF_1 mouse.
[c] In this case, freshly isolated $CD4^+$ T cells from a 5.5-mi-old SNF_1 mouse were used to help in autoantibody production by B cells from a 3.5-mo-old SNF_1 mouse.

was similar in magnitude to the levels found in cocultures without any added Ig (results not shown) (1, 3, 4).

Anti-gp39 Ab blocks lupus nephritis acceleration by pathogenic Th clone in vivo

Unmanipulated SNF_1 female mice uniformly develop severe glomerulonephritis between 6 and 12 mo of age (23), but the representative pathogenic autoantibody-inducing Th clone 3A that is specific for nucleosomes can rapidly accelerate the disease when transferred in vivo into younger mice (8, 9). In accord with those results, herein 3A cells rapidly induced the development of lupus nephritis when injected into 3-mo-old, prenephritc SNF_1 mice that were simultaneously treated with the control Hlg (Table III). These SNF_1 mice began to develop glomerulonephritis by 4 mo of age, i.e., a month after the injection of 3A, and by 5½ mo of age 100% of the mice had developed the disease; similar to SNF_1 mice that are injected with 3A cells alone (8). By marked contrast, the SNF_1 mice that received anti-gp39 Ab treatment in addition to the 3A cells did not develop glomerulonephritis ($p = 0.001$, Fisher's exact test). There were significant reductions in the serum levels of IgG anti-dsDNA and anti-histone/DNA autoantibodies in the anti-gp39-treated mice ($p < 0.01$ and $p <$

Table III. *Anti-gp39 Ab (MR1) blocks lupus acceleration by pathogenic Th clone 3A*

Treatment of 3A-injected Mice[a]	Percent Nephritis[b]	IgG Autoantibodies in Serum[c] U/ml (mean ± SEM)			
		Anti-ssDNA	Anti-dsDNA	Anti-histone	Anti-histone/DNA
Hamster Ig	100	7.94 ± 3.04	2.37 ± 0.43	10.44 ± 1.97	0.96 ± 0.27
Anti-gp39	0	6.31 ± 2.45	0.49 ± 0.16	8.68 ± 2.79	0.05 ± 0.01

[a] Groups of six, 3-mo-old SNF_1 mice received 10^7 cells of the pathogenic autoantibody-inducing Th clone 3A i.v. and five i.p. injections of either the control hamster Ig or the anti-gp39 Ab (MR1) at weekly intervals. The mice were killed for the assays by 5.5 mo of age, i.e. 2.5 mo after inoculation of 3A cells, when all of the hamster Ig-treated mice had developed lupus nephritis.

[b] Glomerulonephritis was graded histologically and by immunofluorescence to be from 3^+ to 4^+ in the hamster Ig-treated mice and these animals had persisted proteinuria of >100 mg/dl for 2 wk before death. The anti-gp39-treated mice at this age had trace proteinuria, normal kidney histology, and their glomeruli showed none to minimal mesangial immune complex deposits by immunoflourescence.

[c] Values are of autoantibody levels in serum diluted 1:100.

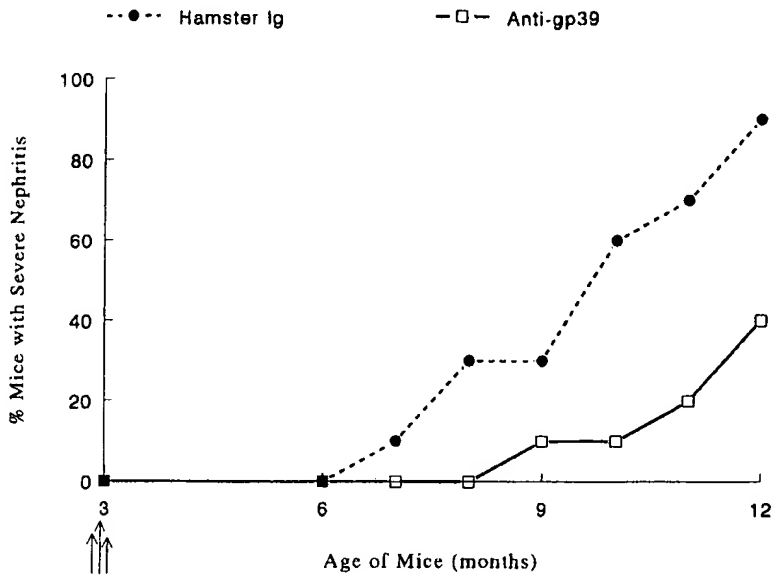

FIGURE 3. Long-term effect of a brief anti-gp39 Ab therapy on spontaneous lupus nephritis. Incidence of severe lupus nephritis in SNF_1 mice (10 animals per group) that received only three injections of anti-gp39 Ab (open squares) or HIg (solid circles) at 3 mo of age.

0.02, respectively); the levels of anti-ssDNA and anti-histone autoantibodies were similar in the two groups of mice (Table III).

A brief anti-gp39 therapy delays the development of spontaneous lupus nephritis

Ten 3-mo-old SNF_1 mice were injected i.p. with anti-gp39 Ab and another 10 mice with HIg every other day for a total of three injections. Three months later, when the mice were 6 mo old, the HIg-injected group began to develop severe glomerulonephritis (Fig. 3), similar to unmanipulated SNF_1 mice (9, 23). In marked contrast, the onset of lupus nephritis was delayed by another 2 mo in the anti-gp39 Ab (MR1)-treated mice and the incidence of glomerulonephritis in this group was significantly lower than the HIg-treated mice when they were 10 to 12 mo of age, i.e., 7 to 9 mo after the anti-gp39 therapy ($p < 0.029$, Fisher's exact test, Fig. 3). Representative examples of kidney histology and immunofluorescence studies from the two groups of mice are shown in Figure 4.

IgG autoantibodies in the serum of these mice were measured after bleeding the mice when they were 7 mo old i.e., 4 mo after the Ab therapy (Fig. 5). Sera from some of the HIg-injected mice that had been killed earlier because of development of severe nephritis were also included. At this timepoint none of the anti-gp39 treated mice had developed nephritis. There was some variation in the levels of autoantibodies in the HIg-treated group of mice, which could be the result of tissue deposition at the time of testing. However, by Mann Whitney U test, the serum levels of three of the four IgG autoantibodies tested were significantly lower in the anti-gp39-treated group as compared with the HIg-treated group: the respective median values for anti-ssDNA were 0.596 vs 4.488 ($p = 0.004$), anti-dsDNA were 0.370 vs 2.245 ($p = 0.005$), and for anti-histone/DNA were 0.120 vs 1.450 ($p = 0.001$).

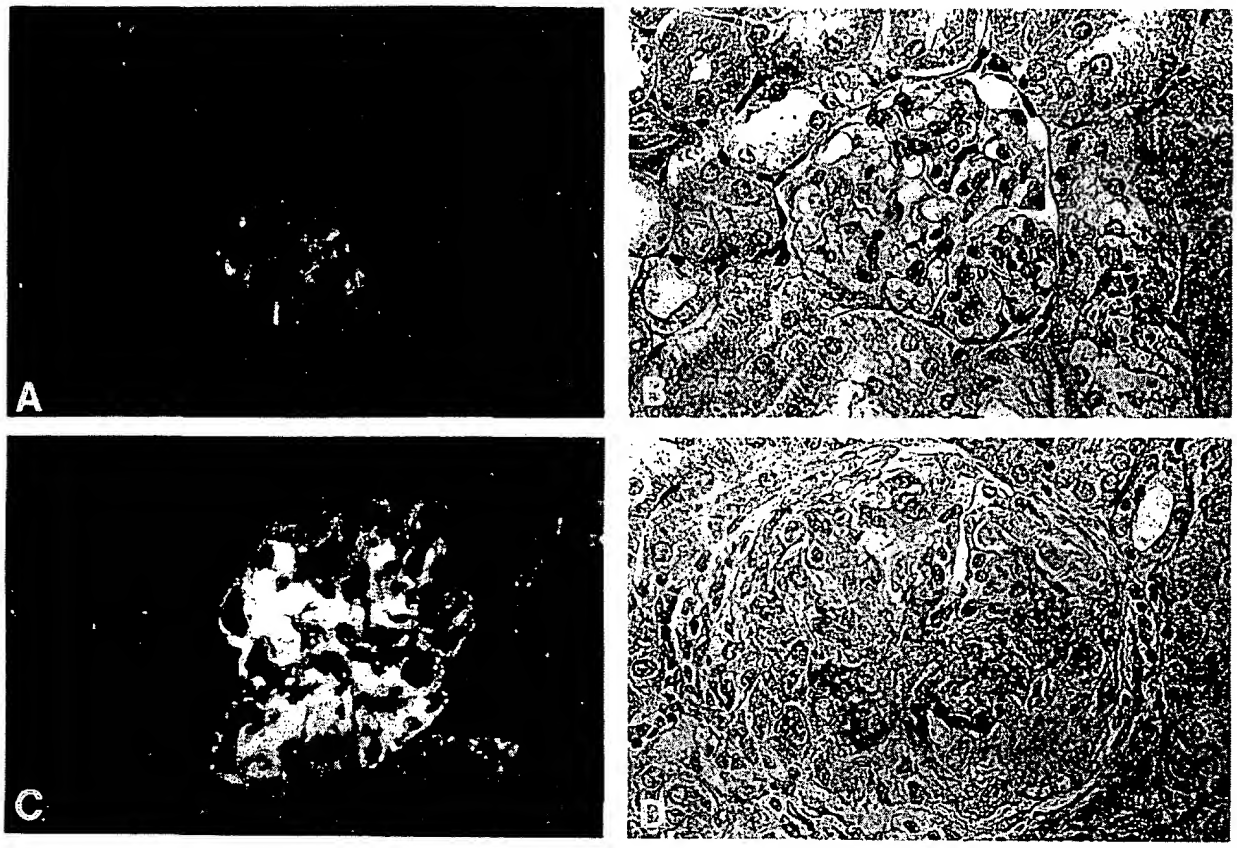

FIGURE 4. Representative immunofluorescence analysis for IgG deposits and histologic evaluation by light microscopy of kidney sections from anti-gp39 Ab-treated (*A* and *B*), and HIg-treated (*C* and *D*) SNF_1 mice at 12 mo of age. Typical kidney section from anti-gp39-treated mouse that had not yet developed lupus nephritis shows minimal IgG deposit in mesangium (*A*) and normocellular glomerulus with open, thin-walled capillary loops (*B*). Control HIg-treated mouse with nephritis shows diffuse and dense granular deposits of IgG in mesangium and capillary walls of enlarged glomerulus (*C*) and histologically the enlarged glomerulus show extensive effacement of capillary loops, hyaline immune-complex deposits, hypercellularity, and crescent formation (*D*); greater than 90% of glomeruli were affected in these kidneys with evidence of vasculitis, dense lymphoid infiltrates and sclerosis of glomeruli. All sections were photographed at ×800.

Long-term immunologic consequences of brief anti-gp39 therapy

Three SNF_1 mice from each of the above two groups (Fig. 3) were tested. Mice from the HIg-treated group that had developed severe nephritis were compared with the age-matched, anti-gp39 (MR1) treated group of mice that had not yet developed nephritis. The mice were 11 to 12 mo old at the time of these assays i.e., 8 to 9 mo after the anti-gp39 or HIg therapy.

Anti-gp39 treated SNF_1 mice that had not developed lupus nephritis at the expected age, still contained pathogenic autoantibody-inducing Th cells in their spleens that could augment the production of pathogenic autoantibodies by ≈7- to ≈54-fold ($p < 0.05$ to $p < 0.01$, Table IV). Indeed, as compared with the HIg-treated mice, Th cells from the anti-gp39-treated mice were capable of inducing higher amounts of IgG autoantibodies to dsDNA and histone/DNA complex when cocultured with the same population of syngeneic B cells in vitro ($p < 0.01$ and < 0.05, respectively, Table IV). Spleens of unmanipulated SNF_1 mice also contained similar levels of pathogenic autoantibody-inducing Th cells at this age (data not shown, see references 1 and 3). The proportion of $gp39^+$, $CD4^+$ T cells in the spleens of mice from the two groups was similar by flow cytometry (20% vs 21% on the average) and by immunohistochemistry (not shown). Moreover, the proliferative responses of splenic T cells to nucleosomes, as assayed by previously described methods (9), were similar in mice from the two groups (data not shown).

Along with the above assays for T cell function, the B cells from the same mice of the two groups described above, were tested for their ability to produce pathogenic autoantibodies in vitro (Table V). Splenic B cells from the anti-gp39-treated mice when cultured by themselves produced much lower levels of IgG autoantibodies than the B cells from age-matched HIg-treated mice: $p < 0.02$ for anti-ssDNA, $p < 0.05$ for anti-dsDNA, $p < 0.001$ for anti-histone, and $p < 0.01$ for anti-histone/DNA. The B cell

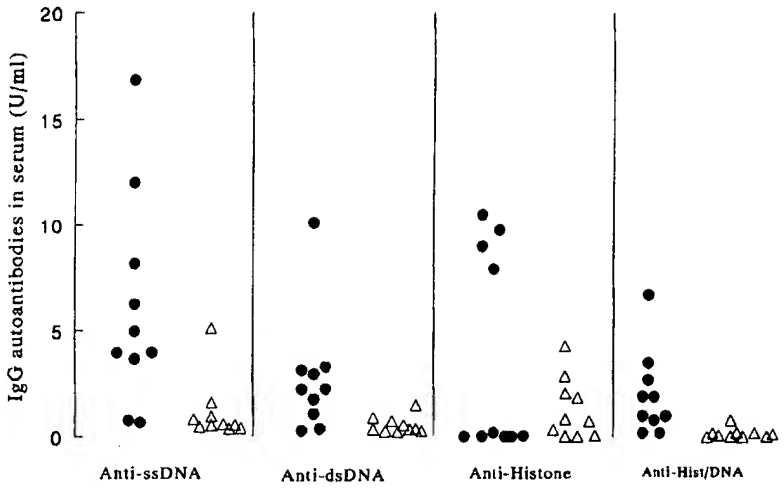

FIGURE 5. IgG autoantibody levels in the serum (at 1/100 dilution) of HIg-treated (solid circles) and anti-gp39-treated (open triangles) SNF_1 mice at 7 mo of age. Each data point represents an individual mouse.

Table IV. *Ability of T cells from anti-gp39 (MR1) Ab-treated mice to provide help for autoantibody production*

Culture Conditions[a]	IgG Autoantibody Production in Culture U/ml (mean ± SEM)			
	Anti-ssDNA	Anti-dsDNA	Anti-histone	Anti-histone/DNA
B_{CNT} alone	0.041 ± 0.008	0.197 ± 0.019	0.037 ± 0.006	0.044 ± 0.005
T_{MR1} + B_{CNT}	1.373 ± 0.249	1.368 ± 0.094	1.985 ± 0.224	1.577 ± 0.117
Increase in folds	33.49	6.94	53.65	35.84
T_{HIg} + B_{CNT}	0.773 ± 0.012	0.586 ± 0.058	1.616 ± 0.090	0.470 ± 0.054
Increase in folds	18.85	2.98	43.68	10.68

[a] A representative of three experiments is shown. B_{CNT} means B cells from a control (CNT), unmanipulated SNF_1 mouse that was 3 mo old. T_{MR1} means $CD4^+$ T cells from a 12-mo-old SNF_1 mouse that was treated with anti-gp39 (MR1) Ab at 3 mo age, and that had not yet developed nephritis. T_{HIg} means $CD4^+$ T cells from a 12-mo-old SNF_1 mouse that had received hamster Ig at 3 mo of age and had developed severe nephritis. Increase in folds means fold increase in IgG autoantibody production in the presence of T cells as compared with B_{CNT} cells cultured alone.

Table V. *Ability of B cells from anti-gp39 (MR1) Ab-treated mice to produce autoantibodies with T cell help*

Culture Conditions[a]	IgG Autoantibody Production in Culture U/ml (mean ± SEM)			
	Anti-ssDNA	Anti-dsDNA	Anti-histone	Anti-histone/DNA
B_{MR1} alone	0.042 ± 0.002	0.294 ± 0.005	0.047 ± 0.002	0.011 ± 0.001
B_{MR1} + T_{CNT}	0.046 ± 0.029	0.311 ± 0.008	0.053 ± 0.032	0.009 ± 0.001
Increase in folds	1.10	1.06	1.13	0.82
B_{HIg} alone	0.263 ± 0.029	0.473 ± 0.016	0.926 ± 0.008	0.081 ± 0.006
B_{HIg} + T_{CNT}	0.922 ± 0.023	2.724 ± 0.114	5.597 ± 0.449	0.773 ± 0.083
Increase in folds	3.51	5.76	6.44	9.54

[a] A representative of three experiments is shown. B_{MR1} and B_{HIg} are B cells from the same 12-mo-old SNF_1 mice shown in Table IV, which had received anti-gp39 (MR1) or hamster Ig therapy, respectively, at 3 mo of age. At the time of this assay, the former animal did not have nephritis, whereas the latter had severe glomerulonephritis. To help the B cells produce autoantibodies in vitro, T_{CNT} were used which are $CD4^+$ T cells from a control (CNT) unmanipulated SNF_1 mouse 6 mo of age.

population from HIg-treated mice that spontaneously produced the autoantibodies in vitro were probably triggered in vivo by Th cells and similar results are found in unmanipulated SNF_1 mice of this age that have developed nephritis (1, 3). Moreover, $CD4^+$ T cells from unmanipulated, 6-mo-old SNF_1 mice that have pathogenic autoantibody-inducing Th cells (1, 3), failed to augment the production of autoantibodies by the B cells from the anti-gp39-treated mice. By contrast, the same T cells could augment further the production of IgG autoantibodies by B cells from the HIg-treated group of mice (Table V). Similar results were obtained when the pathogenic autoantibody-inducing Th cells of clone 3A were cocultured with the B cells from the two groups of mice: B cells from the anti-gp39-treated mice failed to respond to help from 3A in contrast to B cells from the HIg-treated mice (data not shown).

The proportion of $B220^+$ splenocytes was similar in the HIg-treated and anti-gp39-treated group of mice (mean 64% vs 56%, respectively). The values for total IgM (μg/ml) in the serum were, respectively, 1152 ± 49 vs 965 ± 43 ($p < 0.02$) and those for total IgG (μg/ml) were

8713 ± 249 vs 7726 ± 278 ($p < 0.02$) in the HIg-treated group vs the anti-gp39-treated group of mice at killing.

Discussion

These studies show that interaction between gp39 on autoimmune Th cells and CD40 on B cells is important in the spontaneous autoimmune response in SLE, and blocking this interaction at a particular window of time in the natural history of lupus mice produces unexpected long-term benefits. As expected the pathogenic autoantibody-inducing Th clones from these mice, that were $CD4^+$, strongly expressed gp39 on activation. Moreover, Ab to gp39 could block the autoantibody-inducing ability of these Th clones as well as freshly isolated lupus T cells, as tested by coculturing them with syngencic B cells in vitro. Because gp39 is expressed transiently on activated T cells (17, 24), the above observation indicates that the autoimmune Th cells become activated in vitro upon recognizing some autoantigen presented by syngeneic B cells (and other APC) before helping those pathogenic B cells (9). Interestingly, unmanipulated SNF_1 lupus mice had increased numbers of $gp39^+$ T cells in their spleens as early as 1 mo of age, in marked contrast to normal strains (Table I). In normal mice, this type of increase in $gp39^+$ cells is seen only after deliberate immunization (22, 24, 25). In lupus mice with ongoing autoimmune responses, it is expected that spontaneously activated T cells will be detectable (1, 12). However, it is surprising to find such an increase so early at the preautoimmune stage. The SNF_1 female mice spontaneously begin to develop nephritis at 6 mo of age, and pathogenic autoantibodies in their sera become elevated between 3 and 5 mo of age (23, 26). However, just like the early increase in $gp39^+$ T cells, T cells responsive to nucleosomal Ags are also detectable in these lupus-prone mice as early as 1 mo of age (9). Remarkably, upon anti-CD3 activation, a significantly higher proportion of T cells from lupus mice expressed gp39, suggesting an inherent difference from T cells of normal mice.

In a short-term lupus acceleration assay, anti-gp39 could completely block the development of glomerulonephritis induced by transferring a pathogenic autoantibody-inducing Th clone into preautoimmune SNF_1 mice. This experiment was terminated about 1½ mo after the last injection of anti-gp39 Ab, when all of the control, HIg-injected mice developed accelerated nephritis. Previous work involving deliberate immunization of normal mice with conventional Ags showed that anti-gp39 could block primary and secondary Ab responses to T cell-dependent Ags up to 19 days after treatment (25). The $t_{1/2}$ of the anti-gp39 Ab is approximately 12 days and less than 5% of the Ab stays in serum after 21 days (25, 27). In the lupus acceleration assay here, the pathogenic Th clone probably undergoes cycles of activation and gp39 expression after transfer in vivo. Even then, the acceleration of lupus nephritis by the Th clone was blocked up to 45 days after the last administration of anti-gp39 Ab. Significantly, the serum levels of IgG Abs to dsDNA and histone/DNA complex, the autoantibodies most closely associated with lupus nephritis (9, 28, 29), were markedly decreased in the anti-gp39-treated mice.

In addition to blocking lupus acceleration, a brief anti-gp39 treatment also had a profound long-term effect on the development of spontaneous lupus nephritis in SNF_1 mice. Because lupus develops spontaneously in these mice it was difficult to decide when to begin such a brief therapy, the timing of which is much easier to decide in deliberately induced autoimmune diseases (27). Because these mice begin to express elevated levels of IgG autoantibodies in their serum between 3 and 5 mo and begin to develop nephritis by 6 mo of age (9, 23, 26, 30), we decided to inject the anti-gp39 Ab at 3 mo of age when the pathogenic autoantibody-producing B cells are probably just beginning to be expanded by the pathogenic Th cells. Surprisingly, development of lupus nephritis was delayed in the treated mice many months (observed here up to 9 mo after therapy) after the anti-gp39 Ab would have been cleared from the system of these mice. The serum levels of pathogenic autoantibodies were also markedly decreased in the anti-gp39-treated group. Previously, in experiments with conventional Ags or with experimental autoimmune disease deliberately induced by collagen, it was demonstrated that anti-gp39 Ab blocks the Ab responses against these Ags (25, 27). Because these experiments dealt with results in the short term, the presence of the injected anti-gp39 Ab in the mice throughout the assay period could have directly inhibited their immune response.

To determine what caused the sustained beneficial effect in SNF_1 mice here, we first examined the T cells in the anti-gp39-treated mice. Because CD40, the receptor for gp39, is also expressed by non-B cells such as dendritic cells, macrophages, and thymic epithelial cells (18), anti-gp39 could potentially block the development and maturation of pathogenic T cells. Moreover, the interaction between T and B cells is bidirectional, i.e., gp39-CD40 interaction also leads to enhanced CD28-B7 family interaction, thus leading to expansion of not only the B cells, but also the Th cells (18, 31). However, the pathogenic autoantibody-inducing Th cells were left intact and functionally competent in the anti-gp39-treated mice indicating that these Th cells could utilize alternative accessory signals for their expansion. In contrast, the pathogenic B cells of the anti-gp39-treated mice were profoundly affected, even many months after the disappearance of the anti-gp39 Ab from their systems. The anti-gp39-treated mice described here and in other studies (25, 27) did not develop any generalized immunodeficiency or hyper-IgM syndrome that is found in patients with genetically determined defects of gp39 expression (reviewed in Ref. 32). Indeed, the total IgG and IgM levels in the sera of these mice, although comparable to normal strains, were less than the control group of SNF_1 mice. This result could be caused by the significant decrease in the production of autoantibodies in

the anti-gp39-treated group of mice, as these autoantibodies form a substantial proportion of serum Igs in the lupus strains. Moreover, the B cells capable of producing pathogenic autoantibodies were not permanently deleted because some of these mice eventually developed lupus nephritis of delayed onset. Thus, the long-term beneficial effect of anti-gp39 therapy was most probably mediated by checking the expansion of autoimmune memory B cells that are committed to produce the pathogenic autoantibodies. Our observations suggest that the period between 3 and 4 mo of age is an important stage in the natural history of lupus in these mice when the expansion of such pathogenic B cells occurs. These results are consistent with short-term studies with conventional Ags that show an obligatory role of CD40-gp39 interaction in the prevention of apoptosis as well as in the expansion and class-switching of B cells and generation of memory cells in the germinal center (17, 18, 20, 33–36). We have intervened against these events in the ongoing but spontaneous pathogenic autoimmune response of lupus by introducing anti-gp39 at a crucial time point. Our obsevations also indicate that the pathogenic B cells of lupus can be down-regulated and they are not totally autonomous because of some intrinsic defect.

The disease in SNF_1 mice resembles human SLE closely, having very similar immunopathology and female predominance (23, 30). The immunologic mechanisms of the pathogenic autoimmune response in these mice are also very similar to human lupus characterized by the emergence of special Th cells that drive the production of pathogenic anti-DNA autoantibodies (1, 2, 4, 6, 26). Moreover, like the human disease, lupus develops in these mice in the absence of any secondary lupus-accelerating genes like *lpr* (23, 30). Therefore, the long-term benefit of a brief anti-gp39 therapy in these lupus mice is relevant to developing similar treatment in human SLE. Indeed, anti-gp39 therapy was beneficial even at the time early autoimmune abnormalities were manifest in the lupus mice. This suggests that anti-gp39 therapy might be beneficial in newly diagnosed lupus patients or in patients in the early stages of disease flare-up. The efficacy of Ab therapy directed against $CD4^+$ T cells in general has been well demonstrated in murine lupus (37). Herein, the anti-gp39 treatment appears to block mainly the cognate, contact-dependent interaction between pathogenic Th and B cells because they are predominantly activated in SLE. Indeed, chronic anti-gp39 therapy in the (NZB × NZW)F_1 model of lupus suppresses the development of lupus nephritis without causing any generalized immunosuppressive side effects (C. M. Burns and R. J. Noelle, personal communication).

Acknowledgments

We thank Dr. Randolph J. Noelle for his gift of the anti-gp39 Ab (MR1) and for stimulating us to initiate these experiments. We also thank Dr. Jim Sinacore of Northwestern University's Multipurpose Arthritis and Musculoskeletal Disease Center for statistical analysis, Dr. Yashpal Kanwar of the Department of Pathology for evaluation of kidney sections, and Mr. Wei Wu for expert technical assistance.

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Kidney International, Vol. 48 (1995), pp. 458–468

Inhibition of the CD40-CD40ligand pathway prevents murine membranous glomerulonephritis

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Inhibition of the CD40-CD40ligand pathway prevents murine membranous glomerulonephritis. Several forms of glomerulonephritis are induced by antibodies against self or foreign antigens. Normal B lymphocyte antibody production requires T cell costimulatory signals provided in part by T cell surface expression of gp39/CD40ligand (CD40L) that engages the B cell receptor CD40 and induces B cell differentiation and immunoglobulin class switching. We assessed the effect of disrupting the CD40L-CD40 costimulatory pathway, using a CD40-Ig fusion protein, on the development of membranous glomerulonephritis (MGN) in the mouse. MGN is induced by mouse antibodies that recognize and bind to exogenously administered rabbit anti-mouse renal tubular brush border (RbAMBB) IgG immobilized in the glomerular capillary wall. MGN did not occur in nude mice, showing the need of the T cell function. C57Bl/10 mice immunized with RbAMBB and treated with CD40-Ig fusion protein displayed a delayed autologous response and absence of MGN lesions, while control fusion proteins failed to prevent the development of the disease. These observations provide evidence that disruption of the CD40-CD40L costimulatory pathway can prevent the development of MGN by suppressing T cell-dependent antibody production.

Thymus-dependent humoral immune response requires an articulate dialogue between T and B lymphocytes. T cells provide stimulatory signals for B lymphocyte function and antibody production partly through release of soluble cytokines [1] and partly through cell surface receptors that recognize specific ligands on B cells during cell-cell contact [2, 3]. The critical role of physical T cell-B cell interaction in T cell-dependent antibody production has been clearly demonstrated by the observation that combinations of cytokines alone cannot replace physical contact in inducing B cell proliferation and differentiation [4–7]. A variety of other approaches have subsequently confirmed this view [3, 8, 9]. Cell-cell interaction is required for T cell antigen receptor recognition of foreign peptides presented by B cell MHC class II molecules. However, physical association also allows interaction between several counter-receptors that regulate both T and B cell responses [8, 9]. Some of these counter-receptors, including LFA-1-ICAM-1, primarily facilitate intercellular adhesion, whereas others, including CD4-MHC II, LFA3-CD2 and B7-CD28 function as accessory signaling molecules that facilitate mutual T cell-B cell stimulation by reducing the threshold of lymphocyte response to antigen or by transducing stimulatory signals [9–11]. The receptor-ligand pair composed of the B cell-associated receptor CD40, and its T cell ligand CD40L/gp39, has recently been shown to play a key role in the regulation of T cell-dependent antibody production [12–15].

CD40 is a 47 kDa cell surface glycoprotein expressed on most mature and activated B cells [16], related to the TNF receptor family of cell surface molecules [17]. Recently, a natural ligand of CD40, CD40L/gp39, has been identified and found to be a type II integral membrane protein, related to TNF and ligands of CD30, CD27, 4-1BB and Fas [18]. Cell surface expression of CD40L/gp39 in normal T cells is transient, occurring shortly after activation and persisting for only a few hours [19–21]. *In vitro*, both soluble CD40-immunoglobulin fusion protein (CD40Rg, for receptorglobulin) and mAb to CD40L/gp39 block T cell-dependent B cell proliferation, Ig production and Ig-class switching [12, 20, 22]. Recombinant CD40L/gp39 and mAb to CD40, on the other hand, induce these events in the presence of cytokines [20, 22–24]. Interestingly, B cells cultured with IL-4 and triggered by anti-CD40 mAb produce IgE and IgG4, whereas in the presence of IL-10, they preferentially synthesize IgG, IgA and IgM [25, 26]. The physiologic importance of the CD40-CD40L/gp39 interaction was discovered through the elucidation of the underlying defect in a severe form of human immunodeficiency known as the hyper IgM syndrome (HIM). HIM is characterized by overproduction of IgM but absence of IgG, IgA and IgE, and is accompanied by severe recurrent infections. Recent work has shown that T cells from HIM patients express mutated CD40L/gp39 that cannot interact with CD40 on B cells [27–29]. B lymphocytes from these patients are normal and display appropriate immunoglobulin class switching upon stimulation with recombinant wild type CD40L/gp39 [28, 30].

Inappropriate antibody production against self-antigens as well as normal antibody production against foreign antigens that fail to be eliminated from the organism may lead to a variety of disease states. Human idiopathic membranous glomerulonephritis (MGN) is an antibody-mediated disease of unknown etiology that may lead to renal failure. Morphologically, it is characterized by glomerular subepithelial deposits of immune complexes, severe basement membrane thickening, and little or no inflammatory infiltrate [31]. Treatment is typically based on administration of steroids and other immunosuppressive drugs with controversial results [32].

The possibility to genetically manipulate cell surface receptors

Received for publication December 6, 1994
and in revised form February 27, 1995
Accepted for publication February 27, 1995

that mediate cell-cell interactions critical to immune responses offers a potentially powerful means to selectively abrogate undesirable T and/or B cell activity. In the present work, we have explored the possibility to use soluble CD40-Ig fusion protein (CD40Rg) to prevent MGN. Mice injected with purified rabbit-anti-mouse pronase-digested renal tubular brush border IgG (RbAMBB) develop glomerulonephritis that mimicks the morphologic lesions of human MGN, providing a suitable animal model of the disease [33]. We show that early administration of CD40-Ig fusion protein can prevent the development of MGN in the murine model. The ability of CD40Rg to block development of renal lesions provides evidence for CD40-CD40L/gp39 signaling in MGN, and suggests that inhibition of this co-stimulatory pathway may be useful in the treatment of antibody-mediated disease.

Methods

Animals

Eight- to nine-week-old C57Bl/10 and C57Bl/6J nude (nu/nu) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Adult male New Zealand White rabbits were purchased from Charles River Laboratories (Wilmington, MA, USA).

Preparation of RbAMBB

Tubular brush border fraction was extracted from mouse kidney cortices minced and sieved through a 90 μ stainless steel sieve, according to the method of Assmann et al [33]. Sieved material was centrifuged at 400 g for 10 minutes and the cell-pellet discarded. The supernatant was then centrifuged at 78,000 g and the resulting pellet washed three times with distilled water by centrifugation at 78,000 g. The pellet, containing the tubular fraction, was incubated at 37°C for two hours with 30 mg pronase (Calbiochem-Behring Corp., La Jolla, CA, USA) in 60 ml of a 0.8% NaCl-0.02 M Tris HCl buffer, pH 7.8, centrifuged at 100,000 g for one hour and lyophilized. Four rabbits were immunized with 20 mg of pronase-digested tubular brush border fraction in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA) and boosted four weeks later with 10 mg of the same material in incomplete Freund's adjuvant (Sigma). Rabbits were bled two weeks following the boost injection and the IgG fraction from the pooled sera prepared as described [33]. IgG from normal rabbit sera (NRb IgG) were used as a control.

Monoclonal antibodies and immunofluorescence microscopy

Fluorescein (FITC)-conjugated rat anti-mouse CD8a was from Biosource (Camarillo, CA, USA); rat anti-mouse CD4 and rat anti-mouse Thy 1.2 were from Pharmingen (San Diego, CA, USA). Mouse anti-human Fc-specific IgG was from Sigma. Irrelevant IgG_{2b} mAb L14 anti-simian virus 40 large T antigen (gift of Dr. Ed Harlow, Massachusetts General Hospital, Charlestown, MA, USA) was used as control and is referred to as IgG_{2b} mAb. FITC-conjugated goat anti-rabbit IgG, rabbit anti-mouse IgG and rabbit anti-mouse C3 were purchased from Cappel Laboratories (Downington, PA, USA). The goat anti-mouse IgG was absorbed with rabbit IgG, and its specificity was confirmed by absence of binding to kidneys of mice sacrified one day after injection of rabbit anti-rat glomerular basement membrane antiserum [34], while staining with FITC-goat anti-rabbit IgG showed marked linear deposits of rabbit IgG on the glomerular basement membrane.

Table 1. Experimental design

Group	*N* of mice	Passive immunization (day 0)	Treatment	Days of treatment
I	6	none	none	—
II	6	NRb IgG	none	—
III	10	RbAMBB	PBS	0–40
IV	3	RbAMBB	CD8mRg	0–40
V	6	RbAMBB	IgG_{2b} mAb	0–40
VI	7	RbAMBB	CD40mRg	0–40
VII	6	RbAMBB	CD40mRg	10–40
VIII	6	RbAMBB	none	—

Soluble fusion proteins (CD40mRg and CD8mRg) and the irrelevant IgG_{2b} mAb were injected at the dose of 50 μg in 150 μl of PBS on day 0 and every other day subsequently. Group VIII was formed by C57Bl/6J (nu/nu) nude mice. All the animals were sacrificed 40 d after immunization. Abbreviations are: NRb IgG, normal rabbit IgG; RbAMBB, rabbit anti-mouse brush border IgG; CD8mRg, CD8 murine receptorglobulin; CD40mRg, CD40 murine receptorglobulin (additional explanations are in Methods).

All animals were nephrectomized at autopsy and the tissue frozen in liquid nitrogen. Cryostat-cut 5 μm tissue sections were mounted onto slides and stained with FITC conjugated antibody, washed several times in PBS, and examined under an epifluorescence microscope. The intensity of staining was graded on a scale from 0 to ++++ (0, absent; +, minimal and focal in amount and extent; ++, moderate and focal; +++, marked and diffuse; ++++, very marked and diffuse). Semiquantitative photometric immunofluorescence measurement of deposits of rabbit IgG present in the glomeruli was performed on 300 randomly-selected individual glomeruli at a magnification of 600× with a Nikon photometer Model UF x 11A. Fluorescence intensity was expressed as the reciprocal of the exposure time.

Electron microscopy

Small fragments of renal cortex (3 mm^3) were fixed in Karnovsky's paraformaldehyde-glutaraldehyde solution [35], post-fixed in 1% osmium tetroxide and embedded in Epon 812. Thin sections stained with uranyl acetate and lead citrate were studied with a Hitachi electron microscope.

Flow cytometry

FACS analysis was performed on Ficoll-separated splenocytes from all animals included in Groups II-VII for Thy1.2, CD4 and CD8 cell surface expression. In addition, the murine Th2 cell line D10.G4.1 (ATCC, Rockville, MD, USA), unstimulated or stimulated with 10 μg/ml Concanavalin-A (Sigma) for six hours, and unstimulated and 10 μg/ml Concanavalin A-stimulated, Ficoll-separated, splenocytes from normal C57Bl/10 mice, were incubated with CD40Rg for 45 minutes at 4°C in PBS, followed by a secondary FITC-conjugated goat-anti-mouse or goat anti-human affinity purified antibody, washed and subjected to FACS analysis (Becton-Dickinson, Mountainview, CA, USA). To avoid non-specific Fc receptor-dependent binding, splenocytes were preincubated for one hour at 4°C with 100 μg/ml purified mouse IgG (Cappel Laboratories) in PBS, washed and then stained.

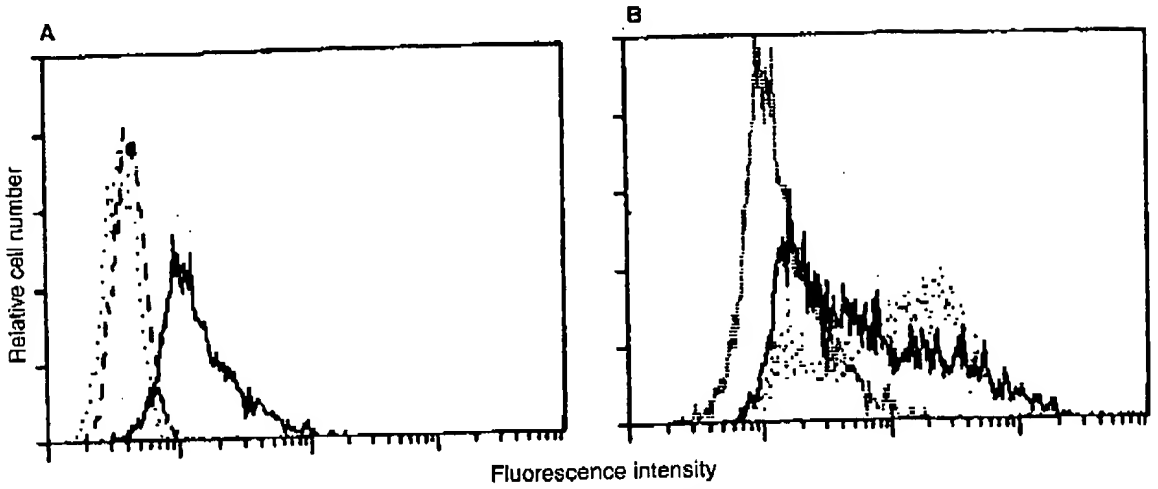

Fig. 1. *Binding of CD40Rg to murine lymphocytes.* (A) Murine D10.G4.1 T cells stimulated with 10 μg/ml Concanavalin A for six hours, were incubated with PBS (dotted line), 10 μg/ml CD40mRg (solid line) or 10 μg/ml CD8mRg (dashed line), followed by an FITC-labeled goat anti-mouse IgG. (B) Unstimulated murine splenocytes were incubated with 10 μg/ml human IgG (broken line) or with 10 μg/ml CD40hRg (solid line), and splenocytes stimulated with 10 μg/ml Concanavalin A for six hours were incubated with CD40hRg (dotted line) followed by an FITC-labeled goat anti-human IgG that does not cross-react with mouse IgG.

Detection of mouse antibodies to rabbit IgG

The autologous antibody response phase of all animals in the experimental groups was assessed by determination of serum mouse anti-Rb IgG levels at days 0, 7, 21 and 40 after immunization in an ELISA. Ninety-six-well microtiter plates were coated with 1 μg/ml of purified rabbit IgG in PBS/0.02% sodium azide overnight at 4°C. The plates were washed with PBS-0.05% Tween-20 and incubated with PBS-2% skimmed milk for 30 minutes at room temperature (RT). After two washes with PBS-0.05% Tween-20, serial 1:50 to 1:500 dilutions of serum samples (100 μl/well) were added. Following an overnight incubation at 4°C, the plates were washed and exposed for four hours to alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), diluted 1:4000. After three washes with PBS-0.05% Tween-20, p-nitrophenyl phosphate (Sigma) dissolved in a 0.1 M glycine/10 mM $MgCl_2$ solution, pH 9.4, was added. Following three hours of incubation at RT, the colorimetric reaction was read at 405 nm in an EL-310 Microplate Autoreader (Biotek Instruments Inc., Winooski, VT, USA).

Proteinuria and PBL counts

Proteinuria at day 40 after immunization was measured using a Protein Assay Kit (Sigma) based on Peterson's modification of the micro-Lowry method with a minimal sensitivity of 50 μg/ml. Samples of peripheral blood were collected in heparinized tubes at the end of the treatment (day 40). Peripheral blood leukocytes (PBL) were counted using a hemocytometer (Fisher Scientific Co., Pittsburgh, PA, USA).

Development and production of soluble recombinant fusion proteins

Soluble receptor globulins were developed by genetic fusion of sequences encoding the extracellular region of murine CD40 to genomic DNA sequences containing exons encoding the hinge, CH2 and CH3 domains of murine IgG_{2b} or human IgG_1 (CD40mRg and CD40hRg, respectively) [36]. Synthetic oligonucleotide primers complementary to the 5′ and 3′ extremities of the nucleotide sequence encoding the extracellular domain of murine CD40 were used to PCR-amplify mouse CD40 from cDNA derived from the WEHI-231 murine B cell line (American Type Culture Collection, Rockville, MD, USA) stimulated for four hours with 5 μg/ml pokeweed mitogen (Sigma). The forward and reverse primers were designed to contain an *Xho*I and a *Bam*HI site, respectively, to facilitate in-frame ligation to Ig expression vectors. Nucleotide sequences of the primers were:

Forward: 5′ CAC GGG CTC GAG ATG GTG TCT TTG CCT CGG CTG TGC GCG CTA TGG 3′

Reverse: 5′ CGC GGG ATC CCG GGA CTT TAA ACC ACA GAT GAC 3′

Thirty amplification cycles at 94°C/1 minute/60°C/2 minutes/72°C/3 minutes were performed using amplitaq polymerase (Perkin-Elmer) and buffers recommended by the vendor. Amplified cDNA was subjected to *Xho*I/*Bam*HI digestion and ligated to *Xho*I/*Bam*HI-cut human IgG_1 expression vector. For insertion into the murine IgG_{2b} expression vector, the CD40hRg construct was digested with *Mlu*I and *Bam*HI and the insert ligated to *Mlu*I/*Bam*HI-cut murine IgG_{2b} expression vector.

Plasmids containg sequences encoding CD40 receptorglobulins bearing murine and human IgG Fc were introduced into COS cells by electroporation at 250 V/960 μF using a Biorad Gene Pulser (Richmond, CA, USA). Serum-free supernatants were collected five to seven days post-transfection and soluble fusion proteins purified on protein A sepharose as previously described [36]. A soluble CD8mRg fusion protein encoded by sequences specific for the extracellular domain of human CD8, previously shown to be non-reactive with murine tissues [37], and murine IgG_{2b} Fc was prepared using the same approach and served as a control. Purified soluble fusion proteins were analyzed by SDS/10% PAGE under reducing conditions. Gels were stained with Coomassie blue.

Table 2. Immunofluorescence findings

		Groups							
		I	II	III	IV	V	VI	VII	VIII
Rb IgG[a]	GPCW	0	0	++++	++++	++++	0	++/+++	0
	M	0	0	0	0	0	0	0	0
	BC	0	0	++	++	++	++	+/++	++
	T	0	0	+/++	+	+	+	+	+
M IgG	GPCW	0	0	++++	++++	++++	0	++/+++	0
	M	++	++	++/+++	++	++	++/+++	++/+++	+++
	BC	0	0	+/0	+/0	0	+/0	+/0	+/0
	T	0	0	+/0	+/0	+/0	0	+/0	0
M C3	GPCW	0	0	+	+/++	+/++	0	+/0	0
	M	++	++	++	++	++	++	++/+++	++
	BC	+++	+++	+++	+++	+++	+++	+++	+++
	T	+++	+++	+++	+++	+++	+++	+++	+++

Abbreviations are: Rb IgG, rabbit IgG; M IgG, mouse IgG; M C3, mouse C3; GPCW, glomerular peripheral capillary walls; M, mesangium; BC, Bowman's capsule; T, tubular basement membrane.

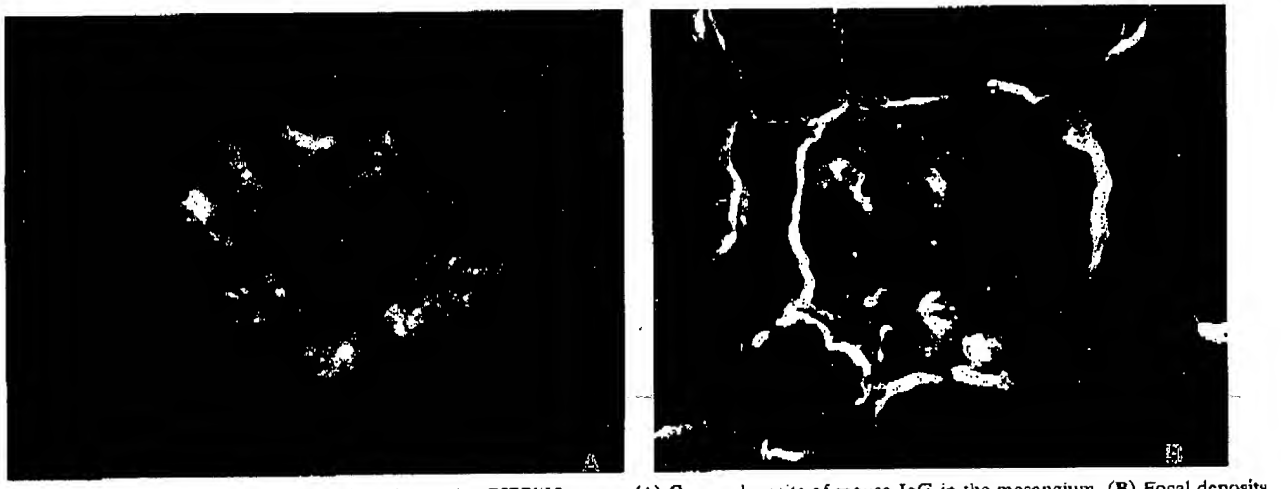

Fig. 2. *Glomerular immunofluorescence findings in a naive C57Bl/10 mouse.* (A) Coarse deposits of mouse IgG in the mesangium. (B) Focal deposits of mouse C3 in the mesangium, Bowman's capsule and tubular basement membranes. ×400.

Induction of MGN and experimental design

C57Bl/10 and C57Bl/6J nude (nu/nu) mice were surgically mononephrectomized under sterile conditions and allowed to fully recover for at least three weeks. On day 0 of the experiment, all animals were immunized with a single injection of 7.5 mg RbAMBB or NRb IgG in 300 μl of PBS into the tail vein. Experimental groups are summarized in Table 1. Mice in Groups III to VI were injected every other day, from day 0 to day 40, with 50 μg CD40mRg, CD8mRg or IgG_{2b} mAb in 150 μl PBS or with 150 μl PBS alone. Mice in Group VII were injected from day 10 to day 40 with 50 μg CD40mRg in 150 μl PBS every other day. All of the mice in groups III to VII received the first ten injections i.v. (tail vein), and the subsequent injection i.p. All animals were sacrificed at day 40. In order to establish whether injections of CD40mRg modified the binding of RbAMBB to the kidneys, additional mice, injected like mice in Groups II and VI, were sacrified at day 1, and the immunofluorescence patterns were compared.

Statistics

Statistical analysis, when applicable, was performed using Statview IV software (Abacus Concepts, Berkeley, CA, USA) on a Macintosh SE computer (Apple Computer, Inc., Cupertino, CA, USA). Differences between groups were compared by one way analysis of variance and unpaired *t*-test.

Results

Characterization of soluble recombinant murine CD40Rg

Murine CD40 extracellular domain-specific sequences were amplified by PCR from WEHI 231 B cell cDNA and ligated to genomic sequences containing murine IgG_{2b} Fc or human IgG_1 Fc exons [36]. Fusion proteins were recovered from COS cells transfected with each of the constructs in a transient expression system, and purified on protein A beads as described previously [36]. Development of CD8mRg was performed by translocating

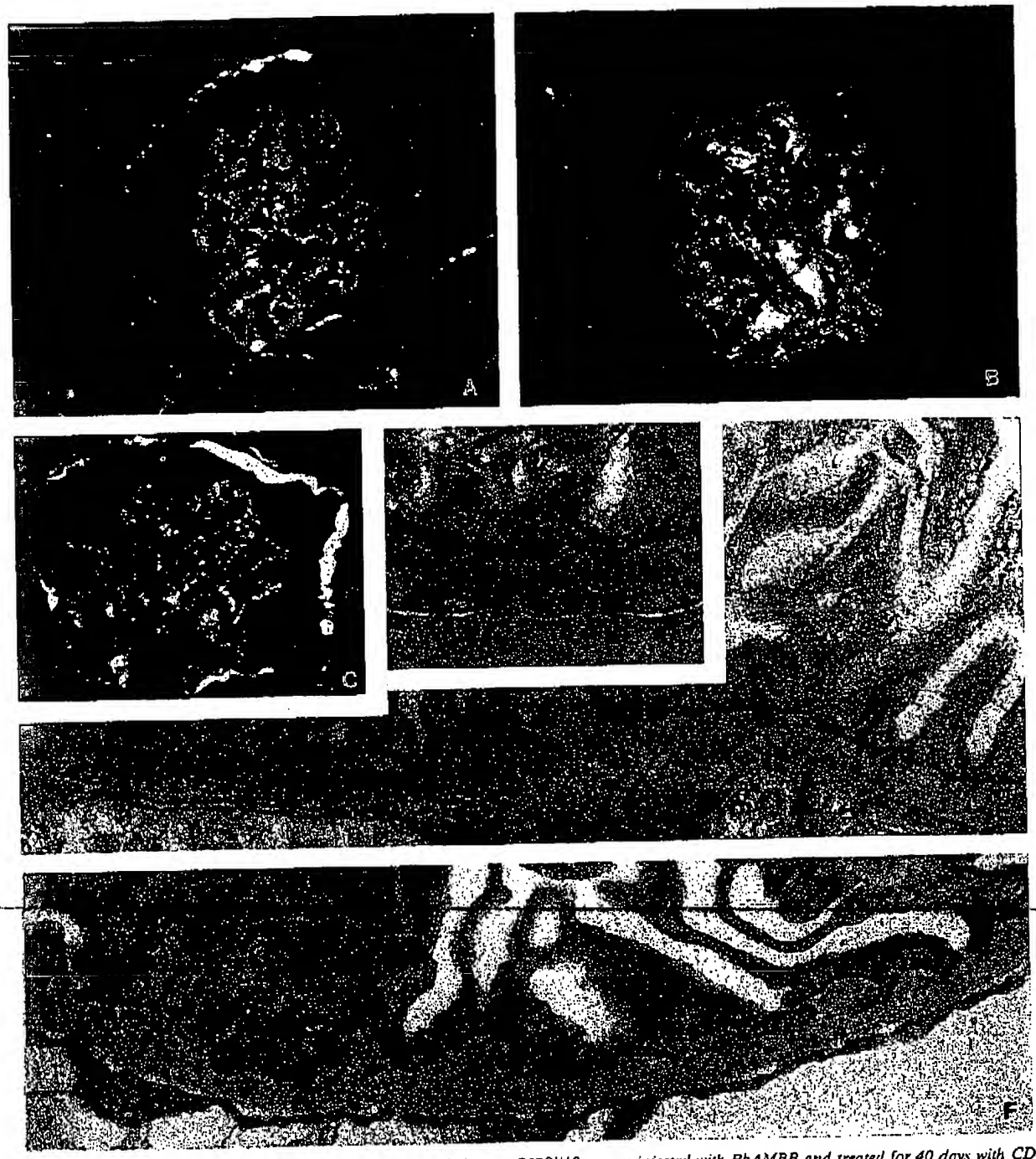

Fig. 3. *Immunofluorescence and electron microscopy of glomeruli from a C57Bl/10 mouse injected with RbAMBB and treated for 40 days with CD8mRg (Group IV).* (A) Granular deposits of rabbit IgG in the peripheral glomerular capillary walls, in Bowman's capsule and in tubular basement membranes. (B) Diffuse, granular deposits of mouse IgG in the peripheral glomerular capillary walls; coarse deposits are also present in the mesangium. (C) Granular deposits of mouse C3 in the peripheral glomerular capillary walls; coarse deposits are also present in the mesangium and in Bowman's capsule. (D and E) Electron micrographs showing deposits of foreign material between the basement membrane and the foot processes (arrow) or in the filtration slits (arrowhead). (F) Electron micrograph showing lesions of the glomerular basement membrane similar to "spikes" of type 2 to 3 human MGN (small asterisks), deposits of foreign material (arrow) and fusion of epithelial foot processes. A–C, ×400; D–F, ×40,000.

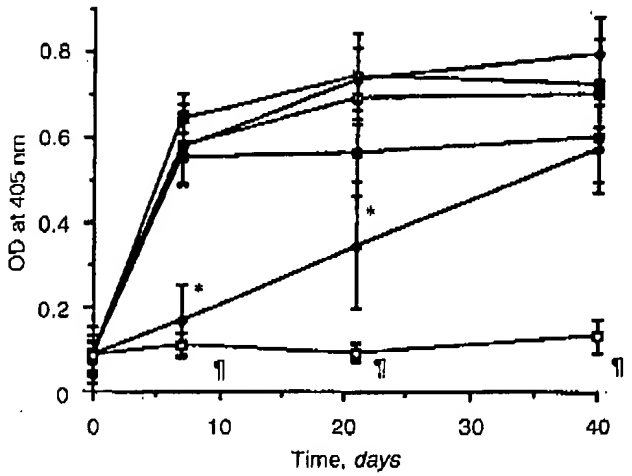

Fig. 4. *Circulating antibodies to Rb IgG detected by ELISA.* At day 7 and 21, the titers of antibodies of Group VI mice were significantly lower than those in Group III and V (*$P < 0.05$). The difference between the titers in Group VIII and those in Groups III and V was always statistically significant (¶$P < 0.05$). Naive mice had an average titer of 0.082 ± 0.020, which was considered as background in the assay. Symbols are: (—□—) Group III, (—◆—) Group IV, (—■—) Group V, (—◇—) Group VI, (—■—) Group VII, (—□—) Group VIII.

the extracellular domain of human CD8 from an expression vector containing human IgG_1 Fc sequences [36] to the corresponding plasmid containing murine IgG_{2b} sequences. SDS/PAGE analysis under reducing conditions showed that purified CD40mRg, CD40hRg and CD8mRg migrated as single bands of 50, 45 and 55 kD, respectively (data not shown).

To determine ligand recognition by CD40mRg, the murine helper T cell line D10.G4.1 was subjected to a six hours stimulation with 10 μg/ml Concanavalin A, and tested for CD40mRg binding by flow cytometry. CD40mRg specifically bound to stimulated but not to unstimulated D10.G4.1 cells (Fig. 1A). Similarly, CD40hRg bound murine Concanavalin A-stimulated splenocytes but reacted weakly with unstimulated splenocytes (Fig. 1B).

Development of MGN

Murine MGN was induced according to the method of Assmann et al [33] by administering a single dose of 7.5 mg of RbAMBB intravenously. To establish the chronology of the lesions, 12 C57Bl/10 mice injected with RbAMBB were subdivided into four groups of three mice each that were sacrificed on days 1, 10, 30 and 40 following injection. Kidney sections from animals in each group were prepared and examined by immunofluorescence. Specimens obtained at days 30 to 40 were also studies by electron microscopy. On day 1, linear deposits of rabbit IgG were seen in the peripheral glomerular capillary walls, in the endothelium of peritubular capillaries and larger vessels and along the brush border of proximal tubules (data not shown). Linear, segmental deposits of mouse C3 were present in the tubular basement membrane and in Bowman's capsule (see Groups I and II). Deposits of mouse IgG were present in the mesangium (see Groups I and II), but were not detectable in the peripheral capillary walls. On day 10, the deposits of rabbit IgG in the peripheral glomerular capillary walls were still linear, and were associated with faint deposits of mouse IgG (data not shown). C3 was present principally in the mesangium. The tubular brush border was no longer stained. On days 30 and 40, diffuse granular deposits of rabbit IgG, mouse IgG and mouse C3 were visible in the peripheral glomerular capillary walls, corresponding to dense deposits in the subepithelial part of the glomerular basement membrane and "spikes" observed by electron microscopy (see Groups III, IV and V). The tubular brush border appeared normal.

Groups I and II

These groups were comprised of normal mice (Group I) and mice injected with normal rabbit IgG (Group II). The experimental design is shown in Table 1. The results obtained by immunofluorescence technique at day 40 are summarized in Table 2. In Groups I and II, that provide negative controls for disease development, coarse deposits of mouse IgG were present in the mesangium (Fig. 2A). Linear segmental deposits of mouse C3 were localized in tubular basement membranes and in Bowman's capsules (Fig. 2B). Some dense deposits were found by electron microscopy in the mesangial matrices. These deposits were present in almost all glomeruli and tubules. In contrast, the peripheral glomerular capillary walls, the interstitium and both small and large vessels were consistently normal. Renal deposits of rabbit IgG were absent. Mouse anti-rabbit IgG were never detectable in sera of Group I animals. Forty days after immunization, the average titer of mouse anti-rabbit IgG in the sera of Group II mice was 0.72 ± 0.14 OD. Proteinuria was 32 ± 4.2 mg/dl and 30 ± 8 mg/dl in Group I and II animals, respectively. PBL count of Group II mice was 7.2 ± 0.6 × 10^3/mm^3 and splenocyte FACS analysis showed 28.5 ± 3.4% Thy 1.2^+, 18.4 ± 2.2% $CD4^+$, 7.5 ± 0.2% $CD8^+$ cells.

Groups III, IV and V

Mice were injected with RbAMBB and treated with PBS (Group III), CD8mRg (Group IV), or the irrelevant mouse monoclonal antibody IgG_{2b} (Group V). Some mice were injected with RbAMBB and PBS, and sacrificed 1, 2 and three days thereafter. On day 1 marked deposits of rabbit IgG were found in glomerular capillary walls, in the brush border and tubular basement membranes. Deposits of mouse C3 were absent. On days 2 and 3 the deposition of rabbit IgG progressively decreased in the glomerular capillary walls and in the brush border, while granular deposits appeared in tubular basement membranes (not shown). At day 40 mice of Groups III, IV and V developed diffuse granular deposits of rabbit and mouse IgG in glomerular peripheral capillary walls, while murine C3 was detectable in only 30% of the animals (Fig. 3A–C). Granular deposits of rabbit IgG, with small amounts of mouse IgG, were present along the basement membranes of proximal tubules and in Bowman's capsules. Electron microscopy revealed small dense deposits in the subepithelial part of the glomerular basement membranes and "spikes" similar to those seen in type 2 to 3 human MGN [31] (Fig. 3D–F). Mesangial deposits were comparable to those observed in Group I and II animals. Antibodies to rabbit IgG became detectable in the serum of these mice at day 7 and reached a peak at day 21 (Fig. 4). Proteinuria was 33.5 ± 9.4 mg/dl (Group III), 29.6 ± 7.3 mg/dl (Group IV) and 27 ± 4.6 mg/dl (Group V) and was comparable to those in normal mice (Group I) and in mice injected with normal RbIgG (Group II). This observation is

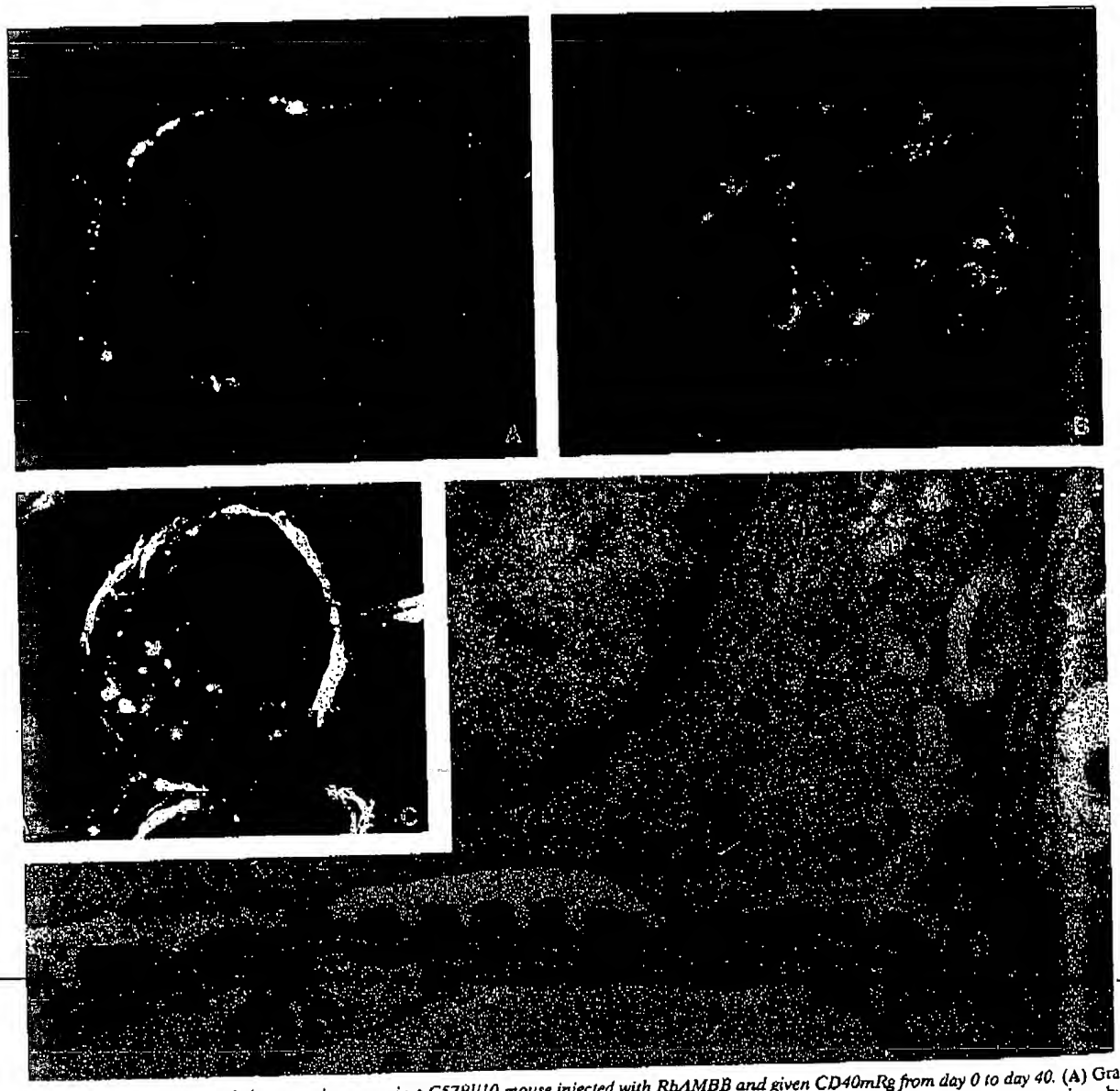

Fig. 5. *Immunofluorescence and electron microscopy in a C57Bl/10 mouse injected with RbAMBB and given CD40mRg from day 0 to day 40.* (A) Granular deposits of rabbit IgG in the Bowman's capsule but not in glomerular peripheral capillary walls. (B) Deposits of mouse IgG in the mesangium, but not in glomerular peripheral capillary walls. (C) Deposits of mouse C3 in the mesangium, in Bowman's capsule and in tubular basement membranes, but not in glomerular peripheral capillary walls. (D) Electron micrograph showing normal glomerular capillary walls. A–C, ×400; D, ×40,000.

consistent with that of Assmann et al [33]. The PBL count was 7.5 ± 1.2 × 10^3/mm^3, 6.8 ± 1.1 × 10^3/mm^3 and 7.0 ± 0.9 × 10^3/mm^3 in Group III, IV and V, respectively. FACS analysis showed the following splenocyte phenotype: Group III, 29.2 ± 4.4% Thy 1.2$^+$, 18.4 ± 4.2% CD4$^+$, 8.3 ± 1.0% CD8$^+$ cells; Group IV, 30.2 ± 2.4% Thy 1.2$^+$, 22.6 ± 3.8% CD4$^+$, 8.0 ± 0.7% CD8$^+$ cells; Group V, 26.8 ± 3.1% Thy 1.2$^+$, 20.9 ± 4.0% CD4$^+$, 6.9 ± 1.2% CD8$^+$ cells.

Group VI

Mice were immunized with RbAMBB and treated with CD40mRg from day 0 to day 40. In mice sacrified one day after injection of RbAMBB and CD40mRg the deposition of rabbit IgG in glomerular capillary walls and tubular brush border was unchanged, as compared to mice injected with RbAMBB and PBS which were sacrificed at the same interval of time (not shown). At day 40 tissue examination by immunofluorescence and electron microscopy revealed normal glomerular peripheral capillary walls in all animals (Fig. 5A–D). The virtual absence of rabbit IgG, compared to mice in Groups III to V, was confirmed by semi-quantitative photometric analysis (Fig. 6). Granular deposits of rabbit IgG were found in the basement membranes of proximal tubules and in Bowman's capsules. Staining for mouse IgG and C3

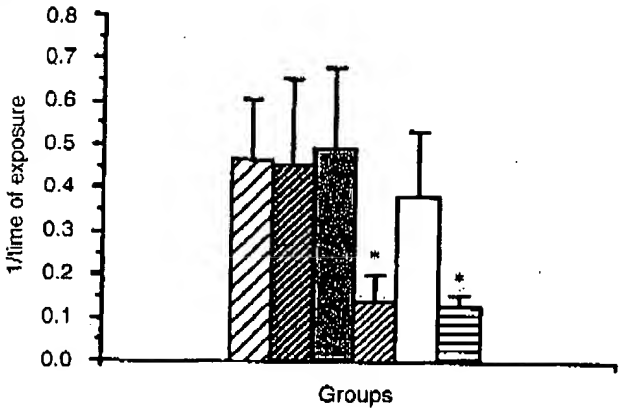

Fig. 6. *Glomerular photometric analysis of the intensity of fluorescence staining for Rb IgG at day 40, expressed as the reciprocal of the exposure time(s).* Values in Groups VI and VIII were significantly lower than those in control Groups III, IV and V (*$P < 0.05$). Symbols are: (▨) Group III, (▨) Group IV, (▨) Group V, (▨) Group VI, (□) Group VII, (▤) Group VIII.

in the mesangium did not differ appreciably from that seen in mice within Groups I to V. During the first three weeks following immunization, mouse anti-rabbit IgG antibody levels were significantly lower than in Group III, IV and V animals (Fig. 4), but rose to comparable titers thereafter. Proteinuria was 27.7 ± 5.3 mg/dl PBL count was 7.1 ± 0.9 × 10^3/mm^3. Splenocyte phenotype was comparable to that of Group III, IV and V animals, with 27.5 ± 3.4% Thy 1.2^+, 18.4 ± 3.6% $CD4^+$, and 7.5 ± 2.2% $CD8^+$ cells.

Group VII

To determine the effect of CD40mRg when administered following disease onset, this group of animals received CD40mRg, their injections beginning on day 10 after RbAMBB immunization when anti-RbIgG antibodies were detectable in serum. Two mice within this group had significantly fewer immune deposits in the peripheral glomerular capillary walls (Fig. 7A and B), whereas glomerular deposits in the other four were comparable to those in Group III, IV and V animals. Although the levels of circulating mouse antibodies to rabbit IgG were lower than in Groups III, IV and V, the difference was marginal (Fig. 4). Proteinuria (29.7 ± 8.4 mg/dl), PBL counts and FACS analysis of splenocyte populations were comparable to those of normal mice.

Group VIII

Nude mice injected with RbAMBB comprised Group VIII. The observations that RbAMBB-induced MGN appears to depend on mouse anti-rabbit IgG production, and that it can be prevented by interfering with T cell-dependent B cell stimulation along the CD40-CD40L/gp39 axis, predict that production of this form of MGN should not be possible in thymus-deficient animals. Accordingly, we administered RbAMBB to a group of nude mice and assessed any resulting glomerular lesions. Deposits of rabbit and mouse IgG and C3 in the glomerular peripheral capillary walls were minimal or absent (Fig. 7 C, D). By electron microscopy a few gross irregularities were seen in the epithelial profile of the glomerular basement membranes, but diffuse subepithelial deposits of foreign material and "spikes" comparable to those seen in mice of Groups III, IV and V, were absent. Mesangial deposits of mouse IgG and C3 did not differ from those seen in naive C57B1/10 and control mice (Groups III, IV and V). Proteinuria was 28.5 ± 8.5 mg/dl. Circulating antibodies to rabbit IgG were not detectable (Fig. 4).

Discussion

Several recent studies have shown that monoclonal antibodies to, and soluble recombinant forms of, specific lymphoid cell surface receptors that mediate cell-cell interaction and participate in the regulation of lymphocyte response to antigenic stimulus, can be used to manipulate the immune response and to control inflammation. Thus, simultaneous administration of anti-ICAM-1 and anti-LFA-1 mAb has been observed to block graft rejection [38] and rapidly progressive glomerulonephritis [39]. Soluble CTLA-4 prolongs allograft [40] and xenograft [41] survival and inhibits rapidly progressive glomerulonephritis [42]. Soluble L- and P-selectin can respectively block peritoneal neutrophil efflux [43] and prevent acute pulmonary inflammation [44]. An anti-CD40L mAb has been effective in preventing collagen-induced murine arthritis [45]. In the present work we have shown that soluble CD40Rg can prevent the development of MGN in the mouse.

CD40Rg binds CD40L/gp39 on the surface of activated T cells, and may thereby prevent CD40L/gp39-CD40 association during cognate T cell-B cell interaction [12]. Since engagement of CD40 by CD40L/gp39 is required for B cell production of antigen-specific IgG, it seems reasonable to suggest that inhibition of CD40L/gp39-CD40 interaction by CD40mRg resulted in the delay of mouse anti-Rb IgG antibody production in the present model. The observed prevention of MGN by CD40mRg was not due to the Fc portion of the fusion protein, since CD8mRg and an irrelevant isotype-matched murine mAb had no effect on antibody deposition in the glomerular capillary walls. Whether binding of CD40mRg to activated T cells *in vivo* results in their opsonization and lysis or only in prevention of CD40L/gp39-CD40 interaction has not been determined. However, the present results are consistent with the notion that CD40mRg induces selective functional inhibition of T cell-B cell interaction without causing leukopenia or even detectable T cell depletion.

MGN may be induced by antibodies binding to constitutive antigens of glomerular visceral epithelial cells or to exogenous antigens immobilized in the subepithelial region of the glomerular basement membrane [46]. RbAMBB recognizes antigens expressed on the surface of murine glomerular endothelial and visceral epithelial cells, including dipeptidyl peptidase IV [47] and aminopeptidase A [48]. The resulting membrane antigen/rabbit IgG complexes are shed between the epithelial cells and the basement membrane [49]. In mice of Groups III, IV and V progressive glomerular disease is induced by a strong and sustained antibody response to rabbit IgG, which probably cross-links immobilized immune complexes to the glomerular basement membrane [50], resulting in progressive enlargement of the complexes that appear as granular deposits in immunofluorescence and electron microscopy. Studies performed with cationic antigens that become immobilized in glomeruli have shown that cross-linking by antibody is a prerequisite for the persistence of these antigens in the subepithelial region of the glomerular basement membrane [51, 52]. We found that the early binding of rabbit IgG to mouse kidneys was not modified by administration

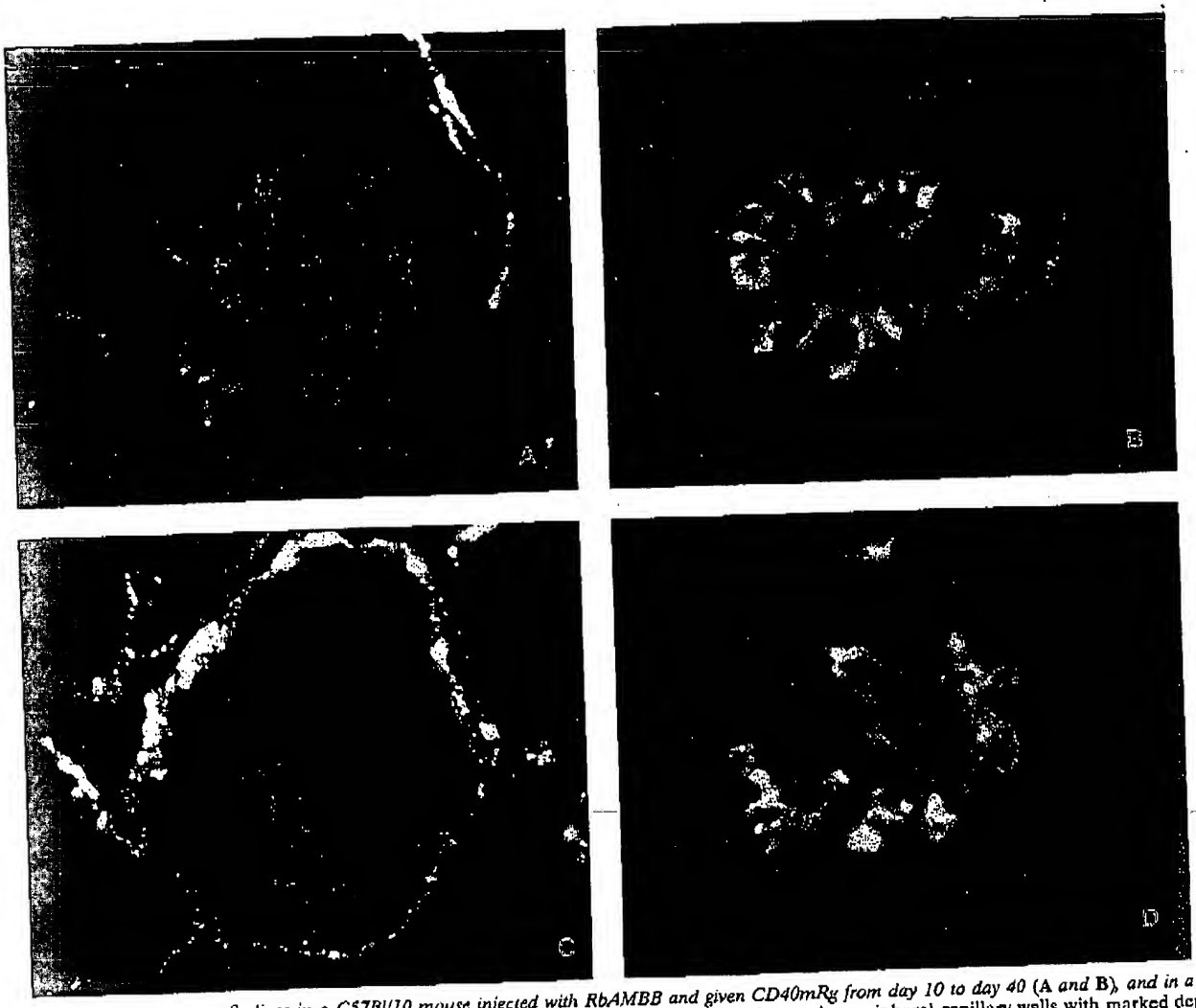

Fig. 7. *Immunofluorescence findings in a C57Bl/10 mouse injected with RbAMBB and given CD40mRg from day 10 to day 40* (A *and* B)*, and in a nude mouse injected with RbAMBB* (C *and* D). (A) Moderate granular deposits of rabbit IgG in glomerular peripheral capillary walls with marked deposits in Bowman's capsule. (B) Minimal to moderate granular deposits of mouse IgG in glomerular peripheral capillary walls and in Bowman's capsule, with coarse deposits in the mesangium. (C) Granular deposits of rabbit IgG in Bowman's capsule and in tubular basement membranes, but not in glomerular peripheral capillary walls. (D) Deposits of mouse IgG in the mesangium, but not in glomerular peripheral capillary walls. ×400.

of CD40mRg. Thus our results indicate that the inhibition of the autologous immune response was responsible for the clearance of immune complexes by glomerular visceral epithelial cells [53], and immune deposits did not develop in the peripheral glomerular capillary walls. This interpretation is supported by the observation that MGN did not occur in nude mice, which lack T cells and cannot mount a T cell-dependent antibody response to rabbit IgG [54].

In contrast, despite suppression of the autologous phase, granular deposits of rabbit IgG were found in the tubular basement membranes and in the Bowman's capsules of mice treated with CD40mRg, as well as in control mice (Groups III, IV and V) and in nude mice (Group VIII). We propose that formation of these deposits does not require cross-linking by mouse IgG, and that they are formed by rabbit IgG and plasma membrane antigens of brush border and basolateral membranes. This hypothesis is in agreement with our previous observations of immune deposits in the basolateral compartments of rabbits [55] and rats [56] injected intravenously with anti-brush border antibodies. The immune deposits in Bowman's capsules are probably formed by complexes of rabbit IgG and plasma membrane antigens shed by glomeruli and reabsorbed by the tubules. Regardless of the nature of the immune deposits, their rapid removal from the peripheral glomerular capillary walls in mice treated with CD40mRg contrasts with their persistance in tubular basement membranes and Bowman's capsules, and suggests different mechanisms of clearance in various parts of the nephron.

While CD40mRg has been observed to be a potent inhibitor of MGN development in the murine model, its effectiveness in reversing established disease was less obvious. Two out of six animals did show a marked reduction in rabbit and murine IgG deposits but only a minimal reduction was seen in four others.

This is most likely a reflection of transient CD40L/gp39 expression on activated T cells, suggesting that timing of CD40mRg administration is of paramount importance. If cognate T cell-B cell interaction, and more specifically, mutual CD40L/gp39-CD40 triggering, has already occurred, CD40Rg may no longer be able to prevent high affinity antibody production. Thus, effectiveness, at least in the present model, appears maximal when CD40mRg and antigen are administered simultaneously. Interestingly, the autologous response phase is inhibited transiently by CD40mRg treatment, and anti-Rb IgG levels in treated animals at 40 days are similar to those of untreated controls. Additional support to this view is provided by a recent study on the effect of a soluble CD40 on antibody response *in vivo* [57]. Possible explanations for the transient inhibitory effect include potential alternative routes for regulation of antibody production that might bypass the CD40-CD40L/gp39 axis, and production of antibodies against an immunogenic CD40mRg epitope, that over time inactivate the fusion protein. Importantly, the presence of mouse anti-Rb IgG antibodies, even in high titers at day 40, can no longer induce MGN, since rabbit IgG has been removed by local cellular mechanisms.

The present study provides direct evidence that timely administration of CD40mRg can efficiently inhibit antibody-mediated glomerular disease. Maximal effectiveness of CD40mRg appears to occur during a narrow window of early immune response, corresponding to cell surface expression of CD40L/gp39 [13, 14, 58]. CD40mRg may therefore provide a valuable reagent in studying the pathogenesis of immune-mediated renal disease. Because of the importance of the time of administration with respect to the disease process, as illustrated by the present model, the effectiveness of CD40Rg in clinical situations may depend in part on diagnostic approaches that are able to uncover early phases of disease activity and exacerbation.

Acknowledgments

This work was supported by National Institutes of Health Grants CA55735 to I.S. and DK-36807 to G.A. I.S. is a Scholar of the Leukemia Society of America. L.B. is recipient of the Dottorato di Ricerche in "Fisiopatologia dell'insufficienza renale," Cattedra di Nefrologia, Università di Parma.

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CD40 Ligand Is Required for Resolution of *Pneumocystis carinii* Pneumonia in Mice[1]

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The role of the CD40-CD40 ligand (CD40L) interaction in resolution of *Pneumocystis carinii* (PC) pneumonia (PCP) was assessed in a PC-infected severe combined immunodeficiency (SCID) mouse reconstitution model using an anti-CD40L mAb to block CD40L. SCID mice infected with PC were reconstituted with unfractionated spleen cells from immunocompetent donors and given either anti-CD40L mAb or an irrelevant control mAb. Mice given the control mAb resolved the PC infection, whereas those given the anti-CD40L mAb did not. That anti-CD40L mAb also inhibited PC-specific IgG production is consistent with the possibility that cognate $CD4^+$ T cell-B cell interactions are important in PCP resolution. The experiment was then repeated, except that the PC-infected SCID mice were reconstituted with purified $CD4^+$ T cells only. Again, the control mAb-treated group resolved the PCP, whereas mice treated with anti-CD40L mAb did not. In the second experiment, inhibition of resolution of PCP in the anti-CD40L mAb group was not the result of blocking $CD4^+$ T cell-dependent activation of PC-specific B cells. The results are consistent with the possibility that resistance to PCP may involve interaction between B cells and $CD4^+$ T cells via the CD40-CD40L pathway. However, results additionally indicate that inhibition of CD40-CD40L interaction ablates resistance to PCP by inhibiting the interaction of T cells with some cell other than B cells. *The Journal of Immunology,* **1995, 155: 3525–3529.**

The expansion of Ag-specific B or T cells is dependent upon the recognition and response to external stimuli in the form of receptor-ligand interactions and cytokine recognition. The detection and response to these stimuli are hypothesized to occur in a chronologic cascade (1). The Ag-specific cognate interaction between the peptide-MHC II complex and the $CD4^+$ TCR triggers this cascade. The initial response is the transient expression of a unique Ag-nonspecific T cell surface ligand known as the CD40L[3] (1, 2). CD40L is recognized and bound by surface CD40 molecules that are constitutively expressed on APC (3). On B cells, this interaction initiates B cell activation and triggers transmission of stimulatory signals from the B7/BB1 surface markers on activated B cells to T cells via their CD28/CTLA1 surface markers. These mutual stimulatory signals, in conjunction with responses to cytokines, elicit the selective expansion of Ag-specific $CD4^+$ T and B cells (4, 5). An analogous cascade of activation events is proposed to take place between monocytes and activated T cells. It is postulated that this series of events is also initiated by the CD40-CD40L interaction and is followed by reciprocating T cell- and monocyte-derived cytokine production (6).

The importance of the CD40-CD40L interaction is manifest by its absence in the X-linked disease hyper-IgM immunodeficiency. In this disease, the impaired T cell function effectuates a failure to undergo isotype switching to IgG, IgA, or IgE, resulting in elevated serum levels of IgM and IgD (7). This impairment has been attributed to amino acid substitutions in the CD40L extracellular domain that prevent its recognition by the CD40 B cell receptor (8). In case studies of patients with hyper-IgM immunodeficiency, an increased susceptibility to recurrent opportunistic infections such as PCP has been noted (7).

The aim of this study was to assess the in vivo role of the CD40L in the resolution of PCP. SCID mice infected with PC were reconstituted with either unfractionated spleen cells or purified $CD4^+$ T cells. These two distinct reconstitution models provided a means to study the role of the CD40L in the resolution of PCP in a B cell-inclusive or B cell-exclusive environment. Treatment of the reconstituted mice with a mAb specifically directed against the murine CD40L resulted in an inhibition of resolution of PCP. These results suggest that although the humoral immune system responds to the PC infection, other effector mechanisms are also involved in the resolution of this disease. However, regardless of the cells involved, the CD40-CD40L interaction plays a necessary role in PCP resolution.

Materials and Methods

Mice

Six- to eight-week-old CB.17 $^{+/+}$ and CB.17 *scid/scid* (SCID) mice were obtained from the Trudeau Institute Animal Breeding Facility (Saranac Lake, NY). A foundation stock of SCID mice was originally obtained from Dr. Leonard Schultz of Jackson Laboratory (Bar Harbor, ME). The SCID mice were bred and housed in microisolator cages containing sterilized food and water. All mice were regularly screened to ensure that no prior exposure to common mouse pathogens had occurred.

Evaluation of PC infection. The severity of a PC infection was assessed by quantifying the number of PC nuclei present in the lungs of infected mice (9). Briefly, the lungs were removed and pushed through a stainless steel screen into 3 ml of HBSS (Life Technologies, Grand Island, NY). An aliquot of the suspension was used to make a cytocentrifuge smear, which was stained with Diff Quik (Baxter, Miami, FL). The number of PC nuclei counted in 30 to 50 oil immersion fields was used to calculate the total number of PC nuclei in the lungs. The limit of detection using this method was $\log_{10}$ 4.1 nuclei/lung.

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Received for publication April 12, 1995. Accepted for publication July 13, 1995.

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[1] This work was supported by U.S. Public Health Service Grant AI-28354 and the F. M. Kirby Foundation, Inc. (Morristown, NJ).

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[3] Abbreviations used in this paper: CD40L, CD40 ligand; PCP, *Pneumocystis carinii* pneumonia; PC, *Pneumocystis carinii*; TBLN, tracheal-bronchial lymph node; EIA, enzyme immunosorbent assay; SCID, severe combined immunodeficiency.

0022-1767/95/$02.00

Table I. *Effect of anti-CD40L mAb treatment on resolution of PCP and PC-specific Ab production in SCID mice reconstituted with unfractionated spleen cells*

Mice-Treatment	Number of PC Nuclei (log_{10})	PC-Specific IgG O.D.	PC-Specific IgM O.D.
Non-reconstituted, untreated	6.44 ± 0.96[a]*	0.001 ± 0.002	0.005 ± 0.008
Reconstituted, control mAb	4.48 ± 0.68	0.200 ± 0.023	0.212 ± 0.087
Reconstituted, anti-CD40L mAb	6.71 ± 0.45**	0.026 ± 0.023***	0.134 ± 0.077****

[a] Numbers are means ± 1 SD, $n = 4$ mice.
* Significantly different from reconstituted SCID mice treated with control mAb, $p = 0.016$.
** $p = 0.002$.
*** Significantly different from reconstituted SCID mice treated with control mAb, $p = 0.008$.
**** Not significantly different from reconstituted SCID mice treated with control mAb, $p = 0.068$.

Natural PC infection of mice

Three-week-old SCID mice were infected with PC by co-housing them with PC-infected SCID mice for up to 6 wk (10). Before their use, the intensity of the PC infection of randomly selected mice was assessed.

Reconstitution of SCID mice with immunocompetent cells

Reconstitutions requiring spleen cells were conducted using 6-wk-old unimmunized male CB.17 +/+ as donor mice. Unimmunized mice were used so that the shift from the primary to the secondary humoral response in the presence or the absence of the anti-CD40L mAb could be detected. Spleens were collected aseptically, gently pushed through stainless steel screens into HBSS, and then triturated with a Pasteur pipette. The spleen cells were washed twice in PBS solution and resuspended to a concentration of 10^8 nucleated cells/ml in PBS. PC-infected SCID mice were given 500 μl of the cell suspension by tail vein injection.

Reconstitutions with TBLN $CD4^+$ T cells were performed using CB.17 +/+ mice that had been previously immunized with PC to maximize the number of recoverable PC-specific cells. The donor animals received intratracheal inoculations according to a previously described technique (11). Two intratracheal inoculations of 10^6 PC in 100 μl of PBS were administered, 8 days apart, followed 3 days later by a 200-μl i.p. injection of CFA (1/1, v/v) containing 2×10^6 PC. TBLN were dissected from their location adjacent to the apex of the lung under sterile conditions 6 days later. The TBLN were gently pushed through stainless steel screens and then triturated with a Pasteur pipette. The cell suspension was depleted of B cells by positive selection for surface Ig-bearing cells using anti-Ig-coated petri dishes. The supernatant was retained, washed in PBS, placed at 4°C, and stained with FITC-conjugated anti-CD4 $F(ab')_2$ for 1 h. The cells were then washed again in PBS, resuspended in PBS-2% fetal bovine serum, and sorted to isolate the $CD4^+$ T cells on a FACStar^{Plus} sorter (Becton Dickinson, Sunnyvale, CA). Recipient SCID mice infected with PC were given 1.2×10^6 sorted $CD4^+$ T cells in 330 μl of PBS by tail vein injection.

Anti-CD40L Ab administration

A hamster-derived mAb, MR1, directed against the CD40L was obtained as a gift from the laboratory of Dr. R. Noelle (Lebanon, NH). Each reconstituted mouse in the anti-CD40L treatment group received 250 μg of this Ab by i.p. injection on days 0, 3, 7, 11, 14, 19, 23, 27, and 31 postreconstitution. The corresponding reconstituted control group was similarly treated, except that the animals received 250 μg of a nonspecific hamster-derived Ig in each injection.

Detection of PC-specific Ab

Serum from each reconstituted mouse was analyzed for PC-specific Ab according to a previously described EIA procedure (12). Briefly, EIA plates (Flow Laboratories, McLean, VA) were coated with 50 μl of a 1/100 dilution of a stock solution containing 1.12 mg/ml of soluble protein made from a partially purified PC preparation. The soluble protein fraction was prepared by removing the cellular debris from the lung homogenate of an infected SCID mouse by centrifugation. The supernatant was then filtered through a 0.22-μm filter, and the remaining protein was quantified using a Lowry Protein Assay Kit (Sigma Chemical Co., St. Louis, MO). PC-specific Ab was detected via the use of an alkaline phosphatase-conjugated goat anti-mouse IgM, IgA, and IgG polyvalent sera. Fifty microliters of a 1/100 dilution of each serum was tested. Control sera for these assays included serum from a nonreconstituted SCID mouse as a negative control and a PC-specific antiserum produced in CB.17 +/+ mice by i.p. injections of PC preparations in IFA (Difco, Detroit, MI). The presence of Ab was revealed by the addition of phosphatase substrate. Absorbance readings were taken at 405 nm using a Titertek Multiskan Plus plate reader (ICN Biomedical, Costa Mesa, CA). Background absorbance readings were automatically subtracted from the sample readings by the program that runs the plate reader.

Flow cytometric analysis

Lung lavage fluids were obtained from the mice as previously described (11). Lymphocytes in the lung lavages and TBLN preparations were stained with the following fluorochrome-conjugated mAb: FITC-anti-CD4, phycoerythrin-anti-CD45RB, phycoerythrin-anti-B220, and FITC-anti-IgG (PharMingen, San Diego, CA). The anti-CD40L Ab was a gift from the laboratory of Dr. R. Noelle (Lebanon, NH). The Ab was biotinylated and detected with streptavidin-conjugated Cy-chrome (PharMingen) by FACScan analysis. The cells were washed and then resuspended in PBS containing 1% BSA-0.1% sodium azide and stained for 30 min at 4°C. Surface marker phenotypes were detected on a FACScan cytofluorometer and analyzed using LYSIS II software (Becton Dickinson) and a Hewlett-Packard 98785A computer (Palo Alto, CA). Control settings for the measured parameters were established before analysis. Forward and side scatter gates were set such that analysis of each sample was confined to only the lymphocyte population in the cell suspension.

Statistics

The significance of difference in the means of the PC and cell numbers from the different treatment groups was calculated using Student's *t*-test. Differences with p values less than or equal to 0.05 were considered significant.

Results

Effect of anti-CD40L mAb treatment on resolution of PCP in unfractionated spleen cell-reconstituted SCID mice

PC-infected SCID mice were reconstituted with unfractionated spleen cells and treated with anti-CD40L mAb or control mAb for 21 days following reconstitution. At that time, the mice were killed, and clearance of the PC infection from their lungs was assessed by enumeration of PC nuclei in lung homogenate smears. As shown in Table I, administration of the anti-CD40L mAb significantly inhibited clearance of the PC. The number of PC nuclei in the anti-CD40L mAb-treated mice was equivalent to that in the unreconstituted SCID mice group, indicating a lack of resolution of the infection. Prior studies using this mAb produced similar results.

PC-specific Ig levels in unfractionated spleen cell-reconstituted SCID mice treated with anti-CD40L mAb

Anti-CD40L mAb treatment or the use of CD40L-deficient mice has demonstrated that a critical role is played by the CD40-CD40L interaction in the generation of T-dependent Ig responses (13, 14). The use of unimmunized spleen cells in this reconstitution model permitted examination of the involvement of the CD40L-CD40 interaction in the primary Ab response and the subsequent isotype switching characteristic of the secondary Ab response. The presence or lack of PC-specific IgG and IgM in the sera of infected

Table II. *Effect of anti-CD40L mAb treatment on numbers of lymphocytes in lung lavage fluids of PC-infected SCID mice reconstituted with unfractionated spleen cells*

Mice-Treatment	Numbers of $CD4^+$ T Cells ($\times 10^4$)		
	$CD40L^+$	$CD45RB^{high}$	$CD45RB^{low}$
Non-reconstituted, untreated	0.03 ± 0.03[a]	0.09 ± 0.09	0.31 ± 0.5
Reconstituted, control mAb	12.54 ± 5.27	2.86 ± 1.43	39.97 ± 18.47
Reconstituted, anti-CD40L mAb	2.03 ± 0.71*	0.73 ± 0.17**	12.69 ± 4.26***

[a] Numbers are means ± 1 SD, $n = 4$ mice.
* Significantly different from reconstituted SCID mice treated with control mAb, $p = 0.008$.
** $p = 0.025$.
*** $p = 0.028$.

Table III. *Effect of anti-CD40L mAb treatment on number of lymphocytes in tracheal-bronchial lymph nodes of PC-infected SCID mice reconstituted with unfractionated spleen cells*

Mice-Treatment[a]	Numbers of $CD4^+$ T Cells ($\times 10^4$)			Number of B Cells ($\times 10^4$)
	$CD40L^+$	$CD45RB^{high}$	$CD45RB^{low}$	$IgG^+/B220^+$
Reconstituted, control mAb	0.69 ± 0.17[b]	1.55 ± 0.05	2.7 ± 0.12	1.45 ± 0.58
Reconstituted, anti-CD40L mAb	0.33 ± 0.14*	0.93 ± 0.53**	0.60 ± 0.32***	0.18 ± 0.08****

[a] Numbers for unreconstituted SCID mice are not shown because lymph nodes cannot be found in these mice.
[b] Numbers are means ± 1 SD, $n = 4$ mice.
* Significantly different from reconstituted SCID mice treated with control mAb, $p = 0.018$.
** Not significantly different from reconstituted mice treated with control mAb, $p = 0.098$.
*** $p < 0.001$.
**** $p = 0.005$.

SCID mice reconstituted with unfractionated spleen cells is shown in Table I at a dilution of 1/100. Titrations significantly beyond 1/100 of PC-specific sera from reconstituted SCID mice possessed Ab activity levels that produced EIA absorbance readings equivalent to background levels, whereas titrations less than 1/100 produced EIA absorbance readings that were attributable to significant nonspecific activity (results not shown). Treatment with anti-CD40L mAb significantly diminished the level of PC-specific serum IgG. However, the PC-specific IgM levels were not significantly reduced relative to those in the control treatment group. These PC-specific Ig levels are representative of those found in earlier experiments with this mAb.

Accumulation of lymphocytes in the lungs and TBLN of unfractionated spleen cell-reconstituted SCID mice following treatment with anti-CD40L mAb

Detection of activated $CD40L^+/CD4^+$ T cells demonstrated that the anti-CD40L mAb treatment did not lyse T cells expressing this marker (Tables II and III). Indeed, others have shown that administration of this mAb in vivo does not physically delete Th cells (14). However, recruitment of activated $CD40L^+$ T cells into the lungs of anti-CD40L mAb-treated mice was significantly diminished relative to that in the control mAb-treated mice (Table II). This significant diminution is reflected by a similar reduction in the number of $CD45RB^{low}$-expressing $CD4^+$ T cells. This marker is equated with the activated subset of the $CD4^+$ T cell population (15). The anti-CD40L mAb treatment also significantly affected accumulation of $CD45RB^{high}$-expressing $CD4^+$ T cells into the lung. Although accumulation of these cells into the lungs was significantly reduced by the anti-CD40L mAb treatment, it was not completely inhibited.

In the TBLN of the anti-CD40L mAb-treated group, the accumulation of both $CD40L^+$- and $CD45RB^{low}$-expressing $CD4^+$ T cells was significantly diminished, whereas the level of accumulation of $CD45RB^{high}$ T cells was not (Table III). The number of B lymphocytes detected in the anti-CD40L mAb-treated group was significantly reduced relative to that in the control mAb-treated group. This diminution in B cell number is reflected in the reduction of PC-specific IgG levels, as shown in Table I.

Table IV. *Effect of anti-CD40L mAb treatment on the resolution of PCP in PC-infected SCID mice reconstituted with fractionated $CD4^+$ T cells*

Mice-Treatment	Number of PC Nuclei ($\log_{10}$)
Non-reconstituted, untreated	6.71 ± 0.26[a]*
Reconstituted, control mAb	4.54 ± 0.70
Reconstituted, anti-CD40L mAb	6.23 ± 0.43**

[a] Numbers are means ±1 SD, $n = 5$ mice.
* Significantly different from reconstituted SCID mice treated with control mAb, $p < 0.001$.
** $p = 0.002$.

Effect of anti-CD40L treatment on PCP resolution in SCID mice reconstituted with purified $CD4^+$ T cells

PC-infected SCID mice were reconstituted with purified $CD4^+$ T cells and killed 35 days later. During this period, some of the mice were treated with the anti-CD40L mAb, and others were treated with the control mAb. This reconstitution model allowed for assessment of the role of the CD40L in a B cell-independent environment. As presented in Table IV, the clearance of PC nuclei from the lungs of infected SCID mice treated with anti-CD40L mAb was significantly inhibited. The number of PC nuclei detected in these mice was equivalent to that found in the untreated/unreconstituted group. As anticipated in this reconstitution model, the levels of PC-specific IgG and IgM were equivalent to background levels, as tested by EIA (data not shown).

Table V. *Effect of anti-CD40L mAb treatment on numbers of lymphocytes in lung lavage fluids of PC-infected SCID mice reconstituted with fractionated CD4+ T cells*

Mice-Treatment	Numbers of CD4+ T Cells (× 10⁴)		
	CD40L+	CD45RB^{high}	CD45RB^{low}
Non-reconstituted, untreated	0.005 ± 0.008[a]	0.007 ± 0.008	0.011 ± 0.007
Reconstituted, control mAb	0.43 ± 0.14	1.31 ± 0.67	4.28 ± 1.47
Reconstituted, anti-CD40L mAb	0.23 ± 0.19*	0.86 ± 0.71**	2.96 ± 1.25***

[a] Numbers are means ±1 SD, $n = 5$ mice.
* Not significantly different from reconstituted mice treated with control mAb, $p = 0.096$.
** $p = 0.334$.
*** $p = 0.164$.

Table VI. *Effect of anti-CD40L mAb treatment on numbers of lymphocytes in tracheal-bronchial lymph nodes of PC-infected SCID mice reconstituted with fractionated CD4+ T cells*

Mice-Treatment[a]	Numbers of CD4+ T Cells (× 10⁴)			Number of B Cells (× 10⁴)
	CD40L+	CD45RB^{high}	CD45RB^{low}	IgG+/B220+
Reconstituted, control mAb	1.04 ± 0.24[b]	3.60 ± 2.22	17.49 ± 8.13	<0.02[c]
Reconstituted, anti-CD40L mAb	0.61 ± 0.57*	1.48 ± 1.04**	8.14 ± 5.5***	<0.02[c]

[a] Numbers for unreconstituted SCID mice are not shown because lymph nodes cannot be found in these mice.
[b] Numbers are means ±1 SD, $n = 5$ mice.
[c] Equivalent to background levels normally detected.
* Not significant from reconstituted control mAb treated mice, $p = 0.164$.
** $p = 0.09$.
*** $p = 0.066$.

Accumulation of lymphocytes in the lungs and TBLN of SCID mice reconstituted with purified CD4+ T cells followed by treatment with anti-CD40L mAb

In this reconstitution model, there were no significant differences in the number of CD4+ T cells, as measured by the expression of their CD40L and CD45RB surface markers, between treatment groups in either the lungs (Table V) or the TBLN (Table VI). The number of B cells detected in TBLN of both groups was so low as to be indistinguishable from normal background levels (Table VI). This would be expected in this reconstitution model and is consistent with the lack of detectable levels of PC-specific IgG or IgM found in these mice (data not shown).

Discussion

The use of separate reconstitution models to address the role of the CD40L-CD40 interaction has shown that this line of cellular communication is shared by both the humoral and cellular branches of the immune system involved in the response to PCP. It was previously demonstrated that resolution of PCP occurred within 21 days after spleen cell reconstitution of PC-infected SCID mice (10). That resolution of PCP after reconstitution with purified CD4+ T cells does not occur until about 35 days (results not shown) suggests that spleen cells discarded during CD4+ T cell purification expedite clearance of PC, but that their presence is not a necessity for PC clearance. This suggests that there are multiple mechanisms used by the host to eliminate the PC infection. In this regard, evidence suggests that Ab, and therefore B cells, may play a role in resistance to PCP (16). Our reconstitution model designed around the use of unimmunized spleen cells is consistent with this hypothesis. A second possible mechanism, independent of B cell function and exemplified in studies previously published, involves resolution of PCP in SCID mice reconstituted with purified CD4+ T cells (17). The use of such a reconstitution model shifts the burden of PC clearance entirely onto the cellular immune system. This permits examination of the possible role of CD40L-CD40 interaction in T cell-macrophage communication during PCP resolution. Results of the present investigation indicate that CD40L-CD40 interactions are necessary for CD4+ T cell-dependent resolution of PCP to occur in either the presence or the absence of B cells.

How the anti-CD40L mAb treatment inhibited resolution of PCP in spleen cell-reconstituted SCID mice is not known. However, we favor the possibility that the anti-CD40L mAb inhibited cognate interactions between CD4+ T cells and target effector cells of the immune system. The inhibition of T cell-B cell communication via the CD40L-CD40 interaction resulting in a diminution of an Ab response could be one of a number of contributing factors to the lack of resolution of PCP. Indeed, those mice reconstituted with unfractionated spleen cells and treated with anti-CD40L mAb demonstrated a failure to undergo IgG isotype switching and had significantly reduced levels of B cell accumulation relative to those treated with control mAb. This effect of anti-CD40L mAb treatment is consistent with the reduced secondary immune response to T-dependent Ag that others have reported in normal mice treated with anti-CD40L mAb (14) and in CD40L-deficient mice (13, 18).

It is also of interest that the administration of anti-CD40L mAb diminished the PC-specific IgM Ab levels, although not significantly, rather than almost ablating them as it did the IgG levels. It has been demonstrated that T-dependent Ig responses are subject to inhibition by anti-CD40L mAb administration and do not occur in CD40L-deficient mice (13, 18). However, host responses to T cell-independent Ag are not subject to CD40L function in that T cell-independent Ag elicit normal primary and secondary serum Ab responses in CD40L-deficient mice (13, 14). It is possible, therefore, that some of the PC-specific IgM response we observed in spleen cell-reconstituted SCID mice was against T cell-independent Ag.

The detection of CD40L+/CD4+ T cells in lungs and TBLN from all reconstituted mice provided evidence that the inhibition of

resolution of PCP by anti-CD40L mAb treatment was not the result of mAb-mediated T cell lysis. During PCP resolution in reconstituted SCID mice, accumulation of lymphocytes in the lungs is attributable to the presence of PC (19). It is possible that the anti-CD40L mAb treatment inhibited $CD4^+$ T cell activation, resulting in lower cell recovery from the lung lavages. The transient expression of CD40L on $CD4^+$ T cells occurs following activation by specific Ag (20). In vitro studies have demonstrated that CD40L is capable of acting as a T cell stimulatory factor following T cell activation, with signals transmitted via the TCR-CD3 complex (21). That $CD40L^+$-, $CD45RB^{low}$-, and $CD45RB^{high}$-expressing $CD4^+$ T cells accumulated in the lungs of spleen-cell reconstituted, anti-CD40L mAb-treated mice in significantly lower numbers than in control mAb-treated mice suggests that T cell activation may have indeed been depressed. The significant reduction in recovery of $CD40L^+$ and $CD45RB^{low}$ T cells as well as the reduced, although not significant, recovery of $CD45RB^{high}$ $CD4^+$ T cells from the TBLN of these spleen cell-reconstituted, anti-CD40L mAb-treated mice would be consistent with this possibility. However, that a number of these $CD4^+$ T cells did accumulate in the lungs of the mice, albeit a lower number, suggests that the activation of $CD4^+$ T cells was not completely inhibited by the anti-CD40L mAb treatment. It is also possible that at least some of the effect of the anti-CD40L mAb treatment occurred downstream of the $CD4^+$ T cell activation, i.e., anti-CD40L mAb treatment may have inhibited trafficking of these T cells into the lung.

How the anti-CD40L mAb treatment inhibited PCP resolution in SCID mice reconstituted with purified $CD4^+$ T cells is not known. However, activated $CD4^+$ T cells did accumulate in the lungs and TBLN of these mice. This suggests that this treatment did not inhibit the activation and accumulation of the $CD4^+$ T cells. Recently, a possible role of the CD40-CD40L interaction in macrophage activation has been revealed. The CD40L has been shown to act as a potent in vitro costimulus of macrophage TNF-α production and as an in vitro induction signal on activated T cell membranes for monocyte IL-1 synthesis (6, 22). Both these cytokines have been proven to play pivotal in vivo roles in the resolution of PCP in reconstituted SCID mice (23, 24). It is plausible, therefore, that the treatment of purified $CD4^+$ T cell-reconstituted SCID mice, with anti-CD40L mAb inhibited resolution of PCP by blocking T cell activation of macrophages through the CD40-CD40L interaction.

In summary, these results are consistent with the possibility that any resistance to PCP originating with cognate $CD4^+$ T cell-B cell communication may involve the CD40-CD40L interaction. However, it was also found that inhibition of the CD40-CD40L interaction ablates resistance to PCP in mice devoid of B cells. Thus, it is possible that inhibition of CD40-CD40L interaction in the presence of B cells actually inhibited an interaction between T cells and some cell other than a B cell, such as a macrophage. Regardless of the cells involved, these results indicate that the CD40-CD40L interaction plays a necessary role in PCP resolution. The results of this investigation are consistent with the possibility that the CD40-CD40L interaction would be important in B cell-dependent resistance as well as crucial for resistance that can be expressed in the absence of B cells. Although the CD40L is tightly regulated and only transiently expressed, the pool of CD40 receptor-possessing cells within the immune system is diverse; thus, the effects of the CD40-CD40L interaction or its inhibition are widespread. Further characterization of the function of the CD40-CD40L interaction in the resolution of infectious diseases that rely on particular effector mechanisms of the immune system needs to be conducted to fully comprehend the effect this receptor-ligand pair has on the immune response.

Acknowledgments

The authors thank Jean Brennan and Mike Tighe for their expert technical assistance. We also thank Dr. Randy Noelle for providing us with the anti-CD40L Ab.

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Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand

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LACK of functional expression of CD40 ligand (CD40L) on T cells results in hyper-IgM syndrome (HIGM1), a human immunodeficiency associated with a severely impaired humoral immune response that is consistent with defects in B-cell responses[1–3]. Patients also succumb to recurrent opportunistic infections such as *Pneumocystis carinii* and *Cryptosporidial* diarrhoea[4,5], suggesting that T-cell functions are also compromised in these individuals, but so far this has not been explained. We have previously shown that mice deficient for CD40L, like HIGM1 patients, show grossly abnormal humoral responses[6]. Here we report that CD40L-deficient mice are defective in antigen-specific T-cell responses. Adoptively transferred antigen-specific CD4$^+$ T cells lacking CD40L failed to expand upon antigen challenge of the recipients, showing that expression of CD40L on T cells is required for *in vivo* priming of CD4$^+$ T cells and therefore for the initiation of specific T-cell immune responses.

To determine whether CD40L influences T-cell responsiveness, we immunized CD40L-deficient and wild-type mice with a protein antigen, keyhole limpet haemocyanin (KLH), and tested their *in vitro* recall proliferative response. A significantly reduced response was observed in CD40L-deficient mice compared with wild-type mice (Fig. 1*a*) and the antigen dose–response curve was shifted by several orders of magnitude. The same defect was demonstrated with two other protein antigens, hen egg lysozyme (HEL) and cytochrome *c* (Cyt-*c*) (Fig. 1*b*, *c*). Furthermore, a dramatic reduction in the production of both interleukin-4 (IL-4) and interferon-γ (IFN-γ) was seen in the CD40L-deficient mice (Fig. 1*d*, *e*), indicating a key role for CD40L in antigen-specific T-cell responses.

The reduced response in CD40L-deficient mice could either be caused by a problem with the antigen-presenting cells (APC) or with T cells. We therefore used APC from wild-type mice to activate CD4$^+$-enriched T cells from CD40L-deficient mice immunized with KLH/CFA (Fig. 2*a*, *b*). Neither wild-type APC (Fig. 2*a*) nor lipopolysaccharide (LPS)-activated B cells (which express co-stimulatory activity and thus might bypass a CD40L requirement) were able to restore the defects (Fig. 2*c*). To address whether APC are intrinsically defective in CD40L-deficient mice, we adoptively transferred wild-type CD4$^+$ T cells to CD40L-deficient mice; these transferred wild-type CD4$^+$ T cells responded vigorously to KLH immunization in the CD40L-deficient recipient (Fig. 2*d*). These observations suggest that the *in vivo* defect lies at the level of the T cells rather than APC. Despite this defect, CD40L-deficient mice do make a weak proliferative response to antigens, suggesting that a CD40L-independent mechanism for T-cell activation must also exist *in vivo*.

To determine whether CD40L-deficient T cells have an intrinsic defect in the ability to proliferate, responses of naive T cells to polyclonal activators were analysed. Naive CD4$^+$ T cells from both wild-type and CD40L-deficient mice proliferated normally in response to anti-CD3, concanavalin A (conA), SEA or SEB (Fig. 3*a*–*d*). To test the *in vitro* primary response to antigen, we crossed CD40L-deficient mice to a Cyt-*c*-specific-TCR transgenic mice, and obtained mice with T cells specific for Cyt-*c* but lacking CD40L. Proliferation of Cyt-*c*-specific CD4$^+$ TCR transgenic T cells from wild-type and from CD40L-deficient mice was identical (Fig. 3*e*). Thus, the intrinsic potential of T cells to respond to antigens is preserved in CD40L-deficient mice. The number and ratios of various T-cell subsets in CD40L-deficient mice are normal, as shown earlier[6]. In addition, the ability to obtain functional Cyt-*c*-specific T cells from

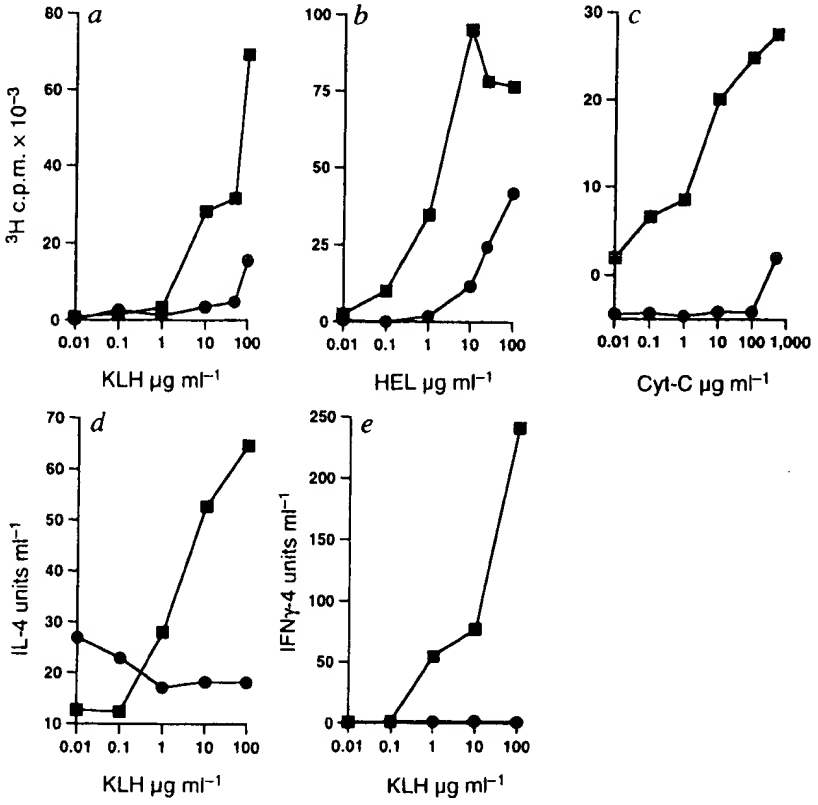

FIG. 1 CD40L mice have impaired lymph node recall proliferative responses to protein antigens. *In vitro* proliferative recall responses of wild-type (■) and CD40L-deficient (●) mice to KLH (*a*), to HEL (*b*), or to Cyt-c (*c*). Production of IL-4 (*d*) and IFN-γ (*e*) by purified CD4$^+$ cells from draining lymph nodes of wild-type and CD40L-deficient mice immunized with KLH/CFA to *in vitro* challenge of KLH.

METHODS. Mice were immunized with 100 μg KLH, HEL or Cyt-c in complete Freunds adjuvant (CFA) in the hind footpads, and 9 days later draining lymph nodes were tested for recall proliferative responses as described[14]. CD4$^+$ T cells were purified from draining lymph nodes as described[15]. Assays for cytokine production by T cells from KLH-immunized mice were conducted by culturing purified CD4$^+$ T cells with APC in the presence of the indicated amount of KLH. After 4 days, supernatant IL-4 and IFN-γ was assayed using an ELISA kit as recommended by the manufacturer (Pharmingen). Experiments were repeated at least three times.

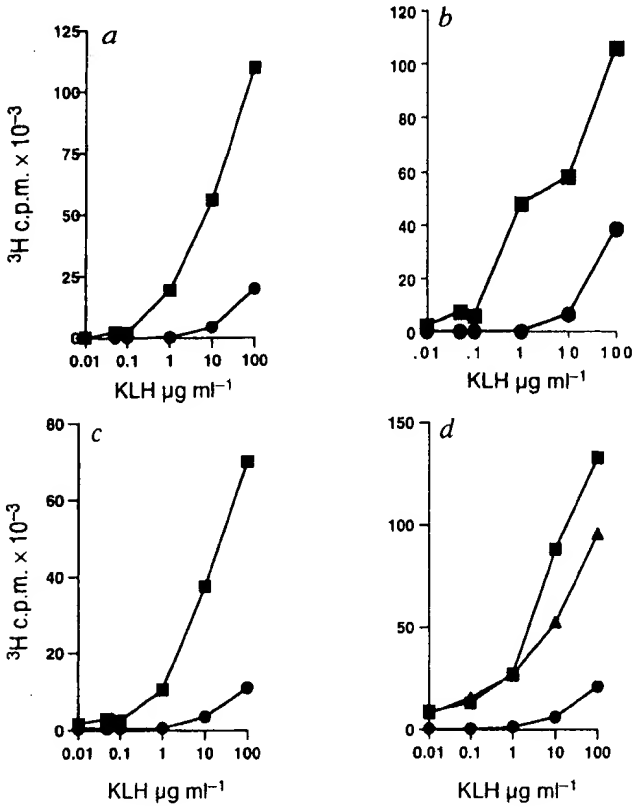

FIG. 2 Lack of response of CD40L-deficient mice to antigen immunization is due to a T-cell defect. Responses are shown of purified $CD4^+$ T cells from KLH/CFA-immunized wild-type mice (■) or CD40L-deficient mice (●) to KLH in the presence of control APC (*a*) or APC from CD40L-deficient mice (*b*); responses are also shown of purified $CD4^+$ T cells to KLH in the presence of LPS-activated splenic B cells as APC (*c*). *d*, Responses to KLH of CD40L-deficient mice (▲) that received 1×10^7 $CD4^+$ T cells i.v. from wild-type mice, and of wild-type and CD40L-deficient mice.

METHODS. Proliferation was measured by culturing 1×10^5 $CD4^+$ T cells from KLH-immunized mice with 5×10^5 irradiated (3,000 R) spleen cells from unimmunized wild-type or from CD40L-deficient mice or with LPS-activated (50 μg ml^{-1} for 48 h) B cells from wild-type mice in the presence of indicated amounts of KLH. Proliferation was determined after 4 d of culture by incorporation of ^{3}H-thymidine.

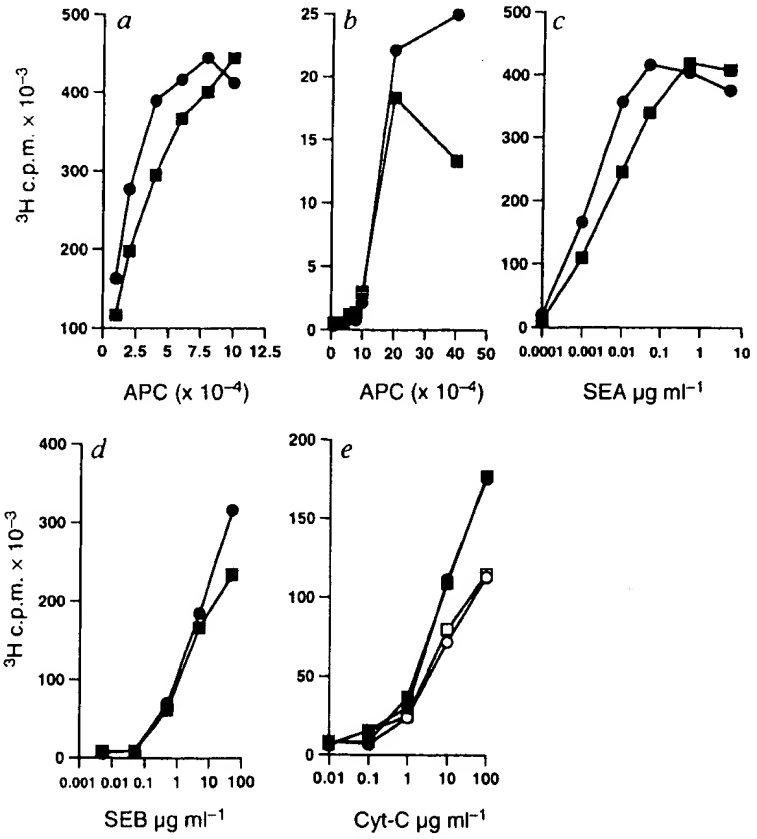

FIG. 3 Naive T cells from CD40L-deficient mice respond normally to antigenic and polyclonal stimuli. Lymph-node T cells purified from unimmunized wild-type (■) or CD40L-deficient (●) mice were cultured in the presence of *a*, conA; *b*, anti-CD3; *c*, SEA; or *d*, SEB. *e*, Lymph node $CD4^+$ T cells purified from Cyt-c-specific TCR transgenic mice lacking CD40L (circles) or control mice (squares) were tested for *in vitro* proliferative responses to Cyt-c in the presence of APC from wild-type mice (filled symbols), and from CD40L-deficient mice (open symbols).

METHODS. Naive lymph node cells were cultured in the presence of wild-type APC at different concentrations in the presence of conA (3 μg ml^{-1}) or anti-CD3 (1 μg ml^{-1}), and in the presence of various concentrations of SEA or SEB. Proliferation was determined after 4 days of culture by incorporation of ^{3}H-thymidine. For testing naive T cells from TCR transgenic mice, female mice homozygous for the CD40L mutation were crossed with Cyt-c-specific-TCR transgenic mice and progeny were screened for the presence of Cyt-c-specific TCR by staining with Vβ3 and Vα11 antibodies. Cyt-c-specific T cells from either CD40L-deficient or from wild-type control mice were cultured in the presence of APC from wild-type or CD40L-deficient mice together with different concentrations of Cyt-c. Proliferation was determined after 4 days of culture by incorporation of ^{3}H-thymidine.

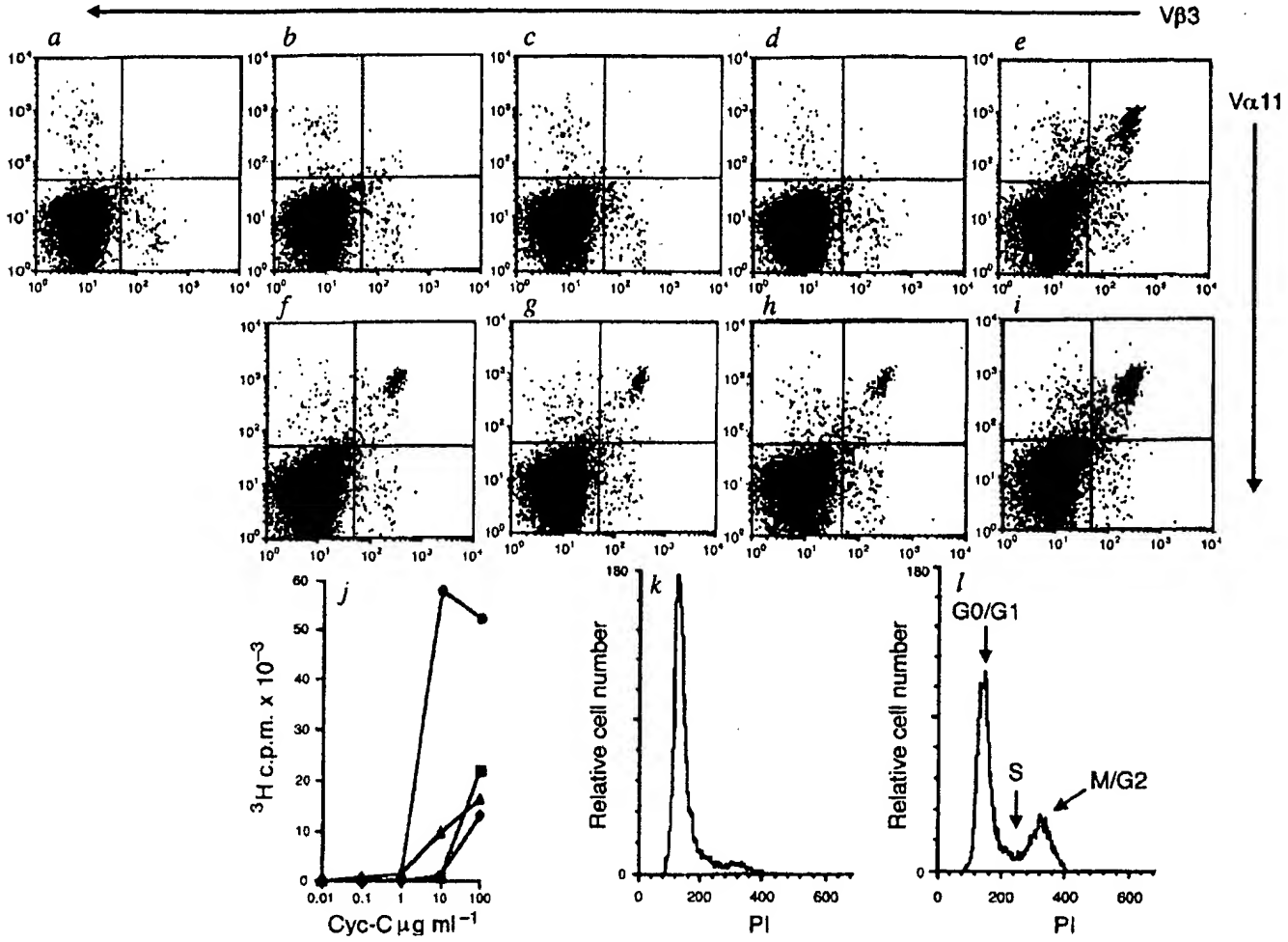

FIG. 4 Adoptive transfer of wild-type of CD40L-deficient Cyt-c-specific-TCR transgenic cells to recipient mice. FACS analysis of draining lymph node cells from mice that did not receive TCR transgenic T cells but were immunized with Cyt-c in CFA (*a*), and from mice that received 5×10^6 CD40L-deficient (*b*, *d*) or wild-type (*c*, *e*) TCR transgenic lymph node cells, and from mice that received 1.1×10^7 CD40L-deficient (*f*, *h*) or wild-type (*g*, *i*) TCR transgenic lymph node cells are shown. Recipient mice were also immunized with Cyt-c in CFA (*d*, *e*, *h*, *i*). *In vitro* recall responses of draining lymph nodes to Cyt-c (*j*) from mice that received 1.1×10^7 CD40L-deficient (◆) or wild-type (■) TCR transgenic lymph node cells, and from mice that were immunized with Cyt-c in CFA and also received 1.1×10^7 CD40L-deficient (▲) or wild-type (●) TCR transgenic lymph node cells. *k*, Flow cytometry plots of cell-cycle analysis for draining lymph node cells by propidium iodide staining for mice that received 1.1×10^7 TCR transgenic lymph node cells lacking CD40L and were immunized with Cyt-c/CFA, and *l*, mice that received 1×10^7 wild-type TCR$^+$ T cells and were immunized with Cyt-c/CFA.

METHODS. Cyt-c TCR transgenic lymph node cells from mice lacking CD40L or from control mice were adoptively transferred i.v. into recipient mice (TCR-negative littermates). A set of recipient mice were also immunized with Cyt-c in CFA (100 μg per mouse in the hind footpads). Five days after transfer, draining lymph node cells were stained with Vβ3 and Vα11 antibodies. Recipient mice that received TCR transgenic lymph nodes but were not immunized with Cyt-c, and mice that did not receive TCR transgenic cells but were immunized with Cyt-c were also tested as controls in the experiments. *In vitro* proliferation of draining lymph node cells was also measured in the presence of Cyt-c. Cell cycle monitoring was as ref. 16.

TCR transgenic CD40L-deficient mice indicates that positive selection of transgenic TCR-specific T cells does not require CD40L.

The failure of the apparently functional T cells from CD40L-deficient mice to respond to antigen stimulation *in vivo* might be explained by an inefficient *in vivo* priming of antigen-specific T cells in CD40L-deficient mice. We therefore adoptively transferred a limiting number of T cells from Cyt-*c*-specific-TCR transgenic mice into recipients immunized with Cyt-*c* (ref. 7) and examined their draining lymph nodes for the presence of transgenic TCR-specific T cells (Fig. 4*a*–*i*) and for Cyt-*c*-specific T-cell responses (Fig. 4*j*) five days after transfer. T cells lacking CD40L failed to expand *in vivo* upon antigen challenge, whereas wild-type cells expanded normally (Fig. 4*a*–*i*). The same results were seen when expansion was monitored 3 days postimmunization (data not shown). To test whether the numbers of T cells from CD40L-deficient mice had decreased after adoptive transfer (for example as a result of apoptosis or a graft-rejection artefact), we repeated the experiments using a higher dose of input T cells. Again, expansion was seen for wild-type CD4$^+$ T cells but not for the CD40L-deficient T cells. The initial population of transferred cells does not, however, diminish, suggesting that CD40L-deficient T cells fail to expand but do not die. Consistent with this, the *in vitro* T-cell response of CD40L-deficient T cells recovered from draining lymph nodes, which is not defective (Fig. 3*e*), can easily be seen for the adoptively transferred CD40L-deficient cells and at an expanded level for wild-type CD4$^+$ T cells (Fig. 4*j*). To determine the stage at which the numbers of CD40L-deficient T cells fail to expand, adoptively transferred Cyt-*c*-specific TCR T cells were analysed for entry into the cell cycle. Wild-type TCR$^+$ T cells enter the cell cycle and substantial numbers of cells (35.5%) were seen in the S/M/

G2 phases of the cell cycles, with the remaining cells in G0/G1 phase (64.5%) (Fig. 4*l*), whereas most (89%) TCR$^+$ cells from CD40L-deficient mice were in G0/G1 phases, with few cells entering S/M/G2 phases (11%) (Fig. 4*k*). This suggests that the defect that leads to failure of the clonal expansion of T cells in CD40L-deficient mice is at an early stage of T-cell activation, preventing T cells from entering the cell cycle.

How then does the CD40–CD40L interaction regulate T-cell priming? This interaction is required for the induction of co-stimulatory activity in B cells[8-10]. We have recently confirmed that wild-type, but not CD40L-deficient T cells can activate co-stimulatory activity in B cells *in vitro*[11]. We believe, however, that this mechanism is unlikely to explain the failure of T-cell priming in CD40L-deficient mice. First, it has recently been shown using B-cell-deficient mice that B cells are not required for T-cell priming to KLH, one of the antigens used in our study[12]. Thus, CD40L activation of B-cell function would not be required for the T-cell clonal expansion we have studied, and therefore could not explain the deficiency in CD40L-deficient animals. Second, the same deficiency in T-cell priming is evident in immunization of myelin basic protein (MBP)-specific TCR transgenic mice with a short peptide of MBP which provokes experimental allergic encephalomyelitis (EAE) in control but not CD40L-deficient animals (I.S.G. and R.A.F., unpublished observations); T-cell response to peptide antigens requires dendritic cell but not B-cell APC function[13]. One possibility still to be investigated is that the CD40–CD40L interaction is necessary for the activation of co-stimulatory activity in APC such as dendritic cells, which are believed to initiate the immune response.

In summary, our results suggest that CD40L is required for *in vivo* clonal expansion of antigen-specific T cells, which in turn may explain the susceptibility of HIGM1 patients to *Cryptosporidial* and *Pneumocystis* infections. Likewise, CD40L-deficient mice have an impaired ability to resist *Leishmania* infection, which is probably contained by T-cell response leading to macrophage activation (L. Soong *et al.*, manuscript submitted). Agonists or antagonists of the CD40–CD40L interaction may eventually have therapeutic benefits: for example, an agent directed at the function of CD40L might be useful in the treatment of autoimmune diseases such as multiple sclerosis. □

Received 24 August; accepted 6 October 1995.

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ACKNOWLEDGEMENTS. I.S.G. and J.X. are Associates and R.A.F. an Investigator of the Howard Hughes Medical Institute. I.S.G. and X.U. contributed equally to this project. We thank K. Bottomly for Cyt-c-specific-TCR-transgenic mice, T. Taylor for help with FACS analysis, and D. P. Grewal for technical assistance.

CD40 ligand-transduced co-stimulation of T cells in the development of helper function

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MICE that lack either CD40[1,2] (expressed on B cells) or CD40 ligand[3,4] (expressed on activated T cells) are able neither to make IgG, IgA or IgE antibody responses, nor to generate germinal centres (the sites of formation of memory B cells). It has been assumed that these lesions were the result of an absence of signals to B cells through CD40. Here we show that the failure to signal T cells through CD40 ligand is an important contributory cause. Administration of soluble CD40 *in vivo* to CD40 knockout mice, restoring the missing signal through CD40 ligand, initiates germinal centre formation. Furthermore, T cells primed in the absence of CD40 (in CD40 knockout mice) are unable to help normal B cells to class switch or to form germinal centres (GC). These results indicate that co-stimulation of T cells through CD40 ligand causes their differentiation into cells that help B cells to make mature antibody responses and to generate memory populations.

To test the ability of T cells primed in the absence of CD40 to help B cells, we immunized CD40 knockout mice, and normal littermates, with keyhole-limpet haemocyanin (KLH). Seven days later, purified T cells from these mice were injected into lightly irradiated CD40 knockout mice, with B cells from non-immune, CD40-expressing, IgH^a congenic donors. These allotype-distinct 'indicator' B cells enable the donor/recipient source of serum antibodies to be determined. Adoptive hosts were immunized with dinitrophenylated KLH (DNP-KLH) (Fig. 1). Ten days after cell transfer, the mice that had received T cells primed in $CD40^+$ environment produced anti-DNP IgM, IgG1, IgG2a and IgG2b antibodies, and formed GC in their spleens. Mice that received T cells primed in CD40 knockout mice produced only IgM antibodies and exhibited no splenic GC (Fig. 1). The IgM and IgG antibodies were of donor IgH^a allotype. The response to DNP-KLH is T-dependent as athymic, nude mice make no anti-DNP or anti-KLH antibodies (results not shown). Thus, T-dependent IgM production can proceed in the absence of CD40 signals to B cells[1,2] and without full-blown helper activity. As full helper activity does not develop over the 14 days in the presence of $CD40^+$ B cells in the adoptive hosts it seems that the CD40–CD40-ligand interaction is crucial during the T-cell priming event and, possibly, cells other than B cells are required.

It is possible that T cells from $CD40^{-/-}$ mice are not tolerant of the $CD40^+$ donor B cells and might therefore reject them. We find no evidence for this as donor IgM^a serum responses were made and IgH^a-positive B cells could be detected in the spleens of recipient mice (results not shown). Likewise, the production of high titres of anti-DNP IgM antibodies indicates that the T cells have not been rendered unresponsive, as recently suggested after immunization of CD40-ligand knockout mice with allogeneic cells[5].

Although the induction of T-dependent IgM antibody responses in $CD40^{-/-}$ mice[1,2] indicates effective T cell priming, we measured the antigen-specific T-cell precursor frequencies, after immunization of the knockout mice with KLH. This revealed variations between $CD40^{-/-}$ mice with a tendency

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CD40-CD40 Ligand Interactions Are Critical in T-B Cooperation but Not for Other Anti-viral $CD4^+$ T Cell Functions

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Summary

CD40-CD40 ligand (CD40L) interaction is required for the generation of antibody responses to T-dependent antigens as well as for the development of germinal centers and memory B cells. The role of the CD40-CD40L interaction in the induction of antigen-specific Th cells and in mediating Th cell effector functions other than cognate help for B cells is less well understood. Using CD40- and CD40L-deficient mice together with lymphocytic choriomeningitis virus and vesicular stomatitis virus as viral model antigens, this study corroborates earlier findings that no Ig isotype switching of virus-specific antibodies was measurable upon infection of CD40- or CD40L-deficient mice. In contrast, in vivo induction of virus-specific $CD4^+$ T cells measured by proliferation and cytokine secretion of primed virus-specific Th cells in vitro was not crucially dependent on the CD40-CD40L interaction. In addition, virus-specific Th cells primed in a CD40-deficient environment, adoptively transferred into CD40-competent recipients, were able to mediate Ig isotype switch. Th-mediated effector functions distinct from and in addition to T-B collaboration were analyzed in CD40- and CD40L-deficient and normal mice: (*a*) local inflammatory reactions upon LCMV infection mediated by LCMV-specific Th cells were not dependent on a functional CD40-CD40L interaction, (*b*) cytokine-mediated protection by $CD4^+$ T cells primed by vesicular stomatitis virus against a challenge infection with recombinant vaccinia virus expressing the glycoprotein of vesicular stomatitis virus was found to be equivalent in CD40L-deficient and normal mice.

Thus, CD40-CD40L interaction plays a crucial role in T-B interactions for Th-dependent activation of B cells but not, or to a much lesser extent, in T cell activation, antigen-specific Th cell responses in vitro, and for interleukin-mediated Th cell effector functions in vivo.

It has been established both in vitro and in vivo that the interaction between CD40 on B cells and its ligand CD40L, which is expressed on activated Th cells, is required for Ig isotype switching, Ig-production and the formation of germinal centers (1–8). In vivo, administration of anti-CD40L antibody or soluble CD40L (9, 10) or CD40-Ig fusion protein (1) as well as studies in CD40- or CD40L-deficient mice (11–13) have shown that the generation of primary and secondary humoral immune responses and the formation of germinal centers to a variety of thymus-dependent antigens were abrogated. Furthermore, patients with hyper-IgM (HIGM)[1] syndrome, a genetic disorder due to mutations in the CD40L gene, exhibit an inability to respond to thymus-dependent antigens and have secondary lymphoid organs which are devoid of germinal centers. Nevertheless, patients suffering from HIGM

[1] *Abbreviations used in this paper:* DNP, dinitrophenol; HEL, hen egg lysozyme; HIGM, hyper-IgM syndrome; KLH, keyhole limpet hemocyanine; LCMV, lymphocytic choriomeningitis virus; LCMV-DNP, DNP covalently coupled to LCMV; OVA, ovalbumin; $VaccG_{IND}$, recombinant vaccinia virus expressing VSV glycoprotein; VSV, vesicular stomatitis virus.

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have normal T cell numbers and are generally not more susceptible to viral infections than healthy individuals (6, 14–18). This may be due to the presence of IgM antibodies and/or CD40L-independent T cell functions.

Since CD40 is not expressed solely on B cells but also on dendritic cells, follicular dendritic cells, monocytes, hematopoietic progenitor cells, and epithelial cells (19–23) CD40-CD40L interaction might be important for the induction and/or effector phase of Th cells in general. This question was adressed in this study. We confirmed here in two infectious virus disease models that a functional CD40-CD40L interaction is not required for T help-independent anti-viral IgM responses but is strictly required for T-dependent Ig class switching of virus-specific B cells. This study additionally investigated the importance of the CD40-CD40L interaction for the induction of Th cell responses and for Th cell effector functions other than cognate T help for B cells.

Using either CD40- or CD40L-deficient mice, Th cell-mediated antiviral immune responses upon infection with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) were investigated. We found that activation and proliferation of virus-specific Th cells did not require a functional CD40-CD40L interaction. Effector functions of Th cells were characterized by (*a*) the analysis of antiviral humoral immune responses, (*b*) Th cell-mediated inflammatory reaction upon LCMV infection, and (*c*) Th cell–mediated antiviral protection against vaccinia virus. Whereas IgM to IgG switch was completely abrogated in both CD40- and CD40L-deficient mice, the two latter effector functions were comparable in CD40-deficient, CD40L-deficient, and normal mice, demonstrating a dichotomy in the role of CD40-CD40L interaction in Th cell effector functions.

Materials and Methods

Mice. Inbred C57BL/6 (H-2^b) mice were obtained from the breeding colony of the Institut für Zuchthygiene (Tierspital Zürich, Switzerland). The generation of mice deficient for CD40, CD40L, or IgM expression has been described previously (11, 12, 24). Mice were bred in a conventional mouse house facility.

Viruses. The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells (ATCC CRL 1; American Type Culture Collection [ATCC], Rockville, MD) with a low multiplicity of infection.

VSV Indiana (Mudd-Summers isolate) seeds, originally obtained from D. Kolakofsky (University of Geneva), were grown on BHK-21 (CRL 8544; ATCC) cells infected at low multiplicity and plaqued on Vero cells.

Vaccinia virus expressing the glycoprotein of VSV was a generous gift of Dr. B. Moss (Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD) (25). Recombinant viruses were grown at low multiplicity of infection on BSC cells and plaqued on BSC cells.

The recombinant baculovirus expressing the LCMV nucleoprotein has been previously described (26). The recombinant baculovirus was derived from nuclear polyhedrosis virus and was grown at 28°C in *Spodoptera frugiperda* cells in spinner cultures in TC-100 medium. Recombinant proteins were produced as previously described (27).

T Cell Proliferation. Mice were immunized intravenously with 200 PFU LCMV-WE. 14 d later, $CD4^+$ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec, Bergisch Gladbach, Germany). 1×10^5 $CD4^+$ T cells were incubated in 96-wells with threefold serial dilutions of either purified, UV-inactivated LCMV (concentrations shown for CD40L−/−, CD40−/−, IgM−/− and C57BL/6 ranged between 0.1 μg/ml and 1 μg/ml), P13 (concentrations shown for CD40L−/−, CD40−/−, IgM−/−, and C57BL/6 ranged between 0.1 μg/ml and 1 μg/ml) or medium only in the presence of 7×10^5 irradiated (2000cGy) C57BL/6 spleen cells for 3 d. Proliferation was assessed by incorporation of [^{3}H]thymidine (25 μCi/well). P13 represents an I-A^b-restricted T cell epitope of the glycoprotein of LCMV which has been described elsewhere (28). We have previously shown that P13 is presented in vivo during a primary immune response against LCMV and that P13-specific $CD4^+$ T cells are induced in vivo (28).

Cytokine Analysis. Supernatants of proliferation assays as described above were analyzed for IL-2 content (24 h after restimulation), IFNγ content (60 h after restimulation), and IL-4 content (60 h after restimulation). IL-2 was determined using the IL-2-dependent cell line CTLL-2. Quantification of viable cells was performed by AlamarBlue™ color reaction (Biosource, International, Camarillo, CA) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore Corp., Bedford, MA) fluorimeter. IFNγ and IL-4 were assessed by ELISA as described (29, 30).

Adoptive Transfer of Primed Th Cells Followed by Challenge with DNP-modified LCMV. CD40-deficient and CD40-competent mice as well as IgM-deficient mice were immunized with 200 PFU of LCMV-WE into both hind foot pads. 18 d later, single cell suspensions were prepared from spleens. 6×10^7 primed spleen cells or an equivalent number of naive cells were transferred intravenously into naive, sex-matched C57BL/6 recipients. Donor cells were pooled from two to three individuals. A few hours later mice were challenged intravenously with 0.4 μg of purified LCMV to which DNP has been covalently coupled (LCMV-DNP) (31). Mice were bled 9 d later and DNP-specific IgG titers were determined by ELISA. The optimal dose for the challenge immunization with LCMV-DNP was determined by in vivo titration of the LCMV-DNP stock in naive versus LCMV-primed mice. The optimal dose was chosen such that only LCMV-primed mice but not naive mice gave rise to a DNP-specific IgG titer.

In addition, the same adoptive transfer experiment was performed using purified, LCMV-primed $CD4^+$ T cells for transfer. CD40-deficient and heterozygous littermates were immunized with 200 PFU LCMV i.v. 14 d later, $CD4^+$ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec) and were used at a purity of at least 95% as checked by FACS analysis. 6×10^6 purified T cells were adoptively transferred into naive C57BL/6 recipients and challenged with 0.4 μg LCMV-DNP. Whole spleen cell transfer from LCMV-primed CD40-deficient mice served as positive control. Mice were bled 10 d later and DNP-specific IgG titers were determined by ELISA.

ELISA. The LCMV nucleoprotein-specific ELISA has been described previously (26). 96-well plates (Petra Plastik, Chur, Switzerland) were incubated with LCMV nucleoprotein (0.01

μg/well) in 0.1 M NaH_2PO_4, pH 9.4, at 4°C. Plates were then preincubated with 2% bovine serum albumin in phosphate-buffered saline for 2 h and washed, and serial dilutions of serum samples (30-fold prediluted) were added to the wells and incubated for 90 min. Plates were washed and incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Sigma Chem. Co., St. Louis, MO). After 90 min, plates were washed and developed with ABTS (5 mg of 2,2'-azino-di-3-ethyl-benzthiazolinsulfonate and 20 μl of H_2O_2 in 50 ml of $NaHCO_3$ [pH 4]). Optical densities were determined at 405 nm.

ELISA-measurement of DNP-specific IgG titers was performed similarly. ELISA plates were coated with 0.25 μg/well of Ovalbumin covalently coupled to DNP (31, 32).

VSV-specific Serum Neutralization Test. Neutralizing titers of sera were determined as described (33). Sera were prediluted 40-fold in supplemented MEM and heat-inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 PFU/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO_2. 100 μl of the serum-virus mixture were transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1 M 2-ME in saline (34).

Assessment of Foot Pad Swelling. Mice were inoculated with 30 PFU of LCMV-WE into both hind foot pads and foot pad swelling was assessed daily with a spring-loaded caliper (35). In vivo anti-CD4 treatment was performed at day 6 and 8 after inoculation using the monoclonal antibody YTS 191.1 (36).

Protection of Mice from Replication of Recombinant Vaccinia Virus. Mice were immunized with 2×10^6 PFU VSV and challenged i.p. 8 d later with 5×10^6 PFU recombinant vaccinia virus expressing VSV-glycoprotein ($VaccG_{IND}$). Vaccinia titers in ovaries were determined 5 d later as described previously (37). Titers are shown as log10 PFU per animal.

Results

Induction of Virus-specific Th Cells. In some experimental systems B cells have been shown to be crucial for efficient T helper cell induction (38–44). In addition, CD40 is not solely expressed on B cells but also on dendritic cells and follicular dendritic cells (6). We addressed therefore the question whether CD40-CD40L interaction or the presence of B cells serving as antigen presenting cells is required for the induction of virus-specific Th cells in vivo. We compared the induction of LCMV-specific Th cells in vivo in CD40-deficient, CD40L-deficient, normal C57BL/6, and IgM-deficient mice. Since it can be expected that B cells do not serve efficiently as APCs in the absence of proper activation and proliferation induced by the CD40-CD40L interaction, IgM-deficient mice that do not have peripheral B cells (24) were included in this comparison. This made possible the distinction between Th cell induction without the CD40-CD40L interaction and Th cell induction without B cells serving as antigen-presenting cells.

Induction of LCMV- or peptide P13-specific Th cells in vivo was comparable in CD40L-, CD40,- and B cell-deficient mice and in normal C57BL/6 mice as defined by specific proliferation in response to both antigens (Fig. 1). These results demonstrate that LCMV-specific Th cells are induced in vivo independent of a functional CD40-CD40L interaction. Since induction of LCMV-specific Th cells in CD40-deficient as well as in CD40L-deficient mice and B cell-deficient mice was similar, Th cells can apparently be stimulated in vivo without crucial involvement of B cells or of a functional CD40-CD40L-interaction after LCMV infection.

In addition, cytokine secretion patterns were analyzed in the supernatants recovered from the proliferation assays performed with LCMV-primed $CD4^+$ T cells from CD40-deficient, CD40L-deficient, or control C57BL/6 mice (Fig. 2). IL-2 and IFNγ were secreted in comparable amounts by $CD4^+$ T cells originating from CD40-deficient, CD40L-deficient, or control mice whereas no IL-4 could be detected in the same supernatants (Fig. 2). Thus, the Th1 cytokine secretion pattern which is normally ob-

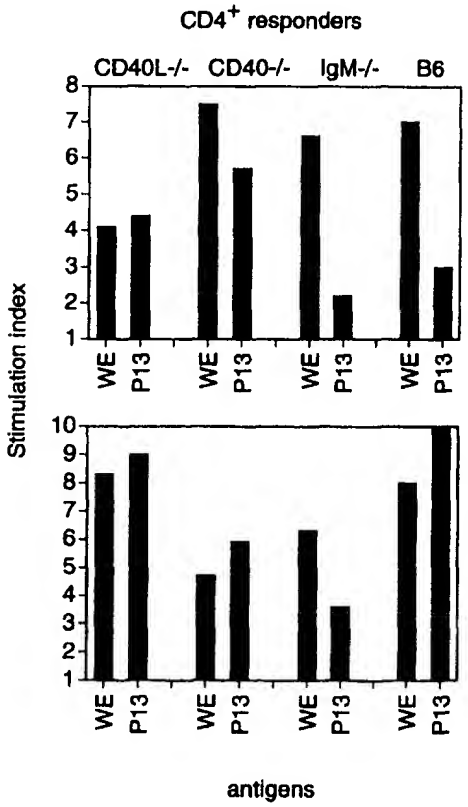

Figure 1. LCMV-specific proliferation of purified Th cells. CD40L-deficient, CD40-deficient, IgM-deficient, and C57BL/6 mice were immunized with LCMV and 14 d later LCMV-specific proliferation of purified $CD4^+$ T cells was determined using either UV-inactivated LCMV (*WE*) or peptide 13 (*P13*) as LCMV-specific antigens (concentrations of stimulating antigens are described in Materials and Methods). P13 is a LCMV-GP-derived peptide recognized by $CD4^+$ T cells. Stimulation indices were calculated in relation to proliferation in medium control. Background cpm counts in medium control were always ~2,000 cpm. Two of four equivalent experiments are shown.

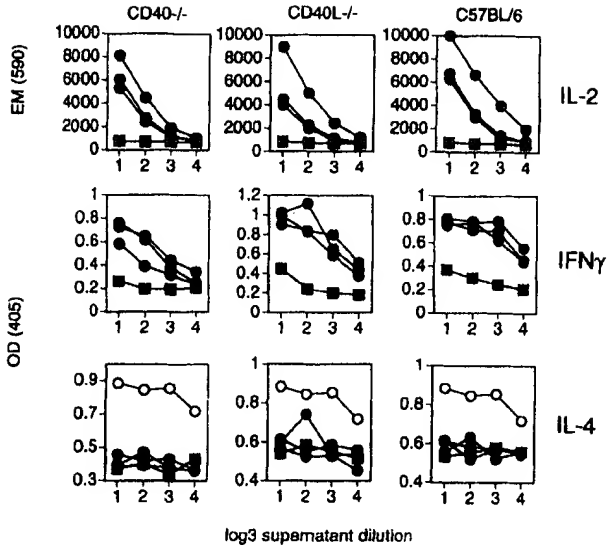

Figure 2. LCMV-specific cytokine secretion. In vivo LCMV-primed CD40-, CD40L-deficient or control $CD4^+$ T cells were restimulated in vitro with the LCMV-glycoprotein-derived I-A^b-binding peptide P13 (*circles*) or without antigen (medium control (*squares*). IL-2 content in the supernatants (5,000 EM U correspond to 30 U IL-2/ml) was analyzed 24 h after restimulation (*top*) whereas IFNγ (OD = 0.5 corresponds to 500 U IFNγ/ml) and IL-4 levels (OD = 0.5 corresponds to 25 U IL-4/ml) were assessed 60 h after restimulation (*middle* and *bottom*). Recombinant IL-4 (*open circles*) served as positive control in the IL-4 ELISA. Each line represents a separate proliferation assay from an individual mouse. One of three equivalent experiments is shown.

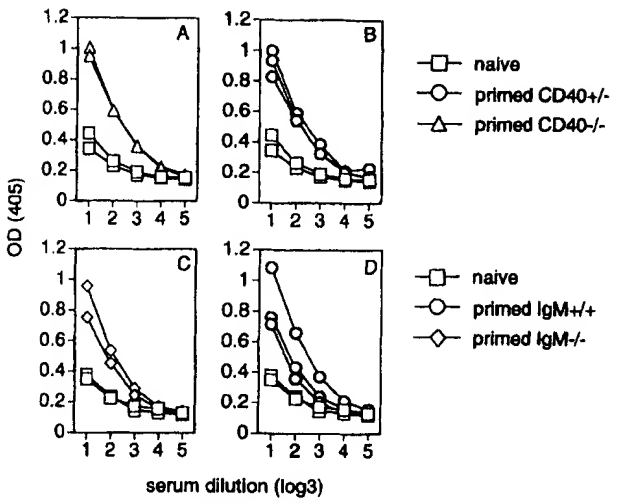

Figure 3. Cognate help to CD40-expressing B cells after adoptive transfer of LCMV-primed Th cells. CD40-deficient mice (*triangles*) and heterozygous littermates (*circles*) as well as IgM-deficient mice (*diamonds*) and control mice (*circles*) were immunized with LCMV. 20 d after infection spleen cells were adoptively transferred into CD40 competent naive recipients and the recipients as well as naive CD40 competent control animals (*squares*) were challenged with LCMV-DNP. After 9 d, DNP-specific IgG titers were determined by ELISA from 30-fold prediluted sera. Each line represents one individual mouse. One of three similar experiments is shown. Transfer of naive $CD4^+$ cells from $CD40^{-/-}$ donors did not result in the generation of significant DNP-specific IgG antibodies (data not shown).

served for LCMV-specific Th cells is not influenced by the absence of functional CD40-CD40L interactions.

In a second experimental approach, induction of LCMV-specific Th cells was assessed in CD40-deficient mice and in heterozygous littermates (both being competent for CD40L on Th cells) by measuring T helper cell function upon transfer of primed Th cells into CD40-competent naive recipients. Thus, CD40L competent cells were primed in a CD40-deficient environment. Adoptive transfer of CD40L-positive, LCMV-primed Th cells into naive recipients with normal $CD40^+$ B cells restores functional T-B cooperation. The conditions for challenging the recipient mice were chosen such that only Th cells already primed in the CD40-deficient donors were able to induce a significant isotype switch in the recipient. To assess the importance of B cells for the induction of Th cells, B cell-deficient mice were included in the experiment. CD40-deficient, B cell-deficient and control mice were infected with LCMV and 18 d after infection spleen cells were transferred into naive recipients. This was followed by a challenge immunization with LCMV-DNP. DNP-specific IgG antibodies were only significantly induced after challenge if LCMV-specific Th cells had been induced in the donor animals before transfer (Fig. 3). Since B cells in the recipients express CD40, equivalent DNP-specific IgG titers in recipients that received primed Th cells from immunized CD40-deficient mice or from heterozygous littermates revealed that induction of LCMV-specific Th cells was not dependent on a functional CD40-CD40L interaction in vivo (Fig. 3, *A* and *B*). Similarly, equivalent IgG titers in recipients that received Th cells from LCMV-immunized B cell-deficient mice or B cell-competent C57BL/6 mice indicated that induction of LCMV-specific Th cells was not dependent on the presence of B cells serving as antigen presenting cells (Fig. 3, *C* and *D*).

To confirm that the transferred $CD4^+$ T cells were alone responsible for the enhanced DNP-specific IgG titers, purified $CD4^+$ T cells from LCMV-primed CD40-deficient mice or heterozygous littermates were transferred into naive C57BL/6 recipients which were challenged by injection of LCMV-DNP. DNP-specific IgG titers were determined 10 d later (Fig. 4, *A* and *B*). In addition, transfer of unseparated spleen cells from LCMV-primed CD40-deficient donors into naive C57BL/6 recipients served as positive control (Fig. 4 *C*), whereas unmanipulated, unprimed C57BL/6 mice served as negative control. Both $CD4^+$ T cells originating from either LCMV-primed CD40-deficient mice or heterozygous littermates were able to comparably provide help for CD40-competent B cells (Fig. 4, *A* and *B*), albeit whole spleen cell transfers resulted in a more pronounced enhancement of DNP-specific IgG titers as compared to purified $CD4^+$ T cells.

Thus, three independent assays showed that the induction of LCMV-specific Th cell responses was not limited by CD40-CD40L interactions and was independent of B cells acting as critical APCs.

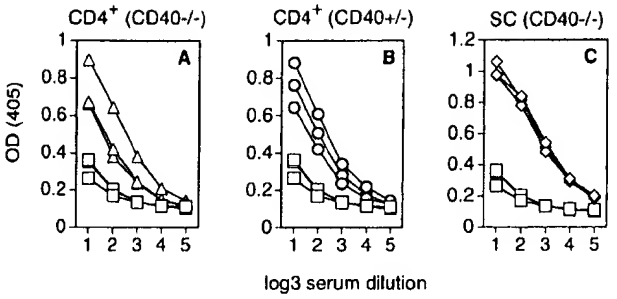

Figure 4. Cognate help to CD40-expressing B cells after adoptive transfer of purified, LCMV-primed $CD4^+$ T cells. CD40-deficient mice (*triangles*) and heterozygous littermates (*circles*) were immunized with 200 PFU LCMV i.v. 14 d later $CD4^+$ T cells were purified and adoptively transferred into CD40-competent, naive recipients (*A* and *B*). In addition, spleen cells from LCMV-primed CD40-deficient donors (*diamonds*) were transferred into CD40-competent naive recipients (*C*). All recipients as well as naive control animals (*squares*) were challenged with LCMV-DNP and 10 d later DNP-specific IgG titers were determined in 90-fold prediluted sera. Each line represents an individual recipient. One of two similar experiments is shown.

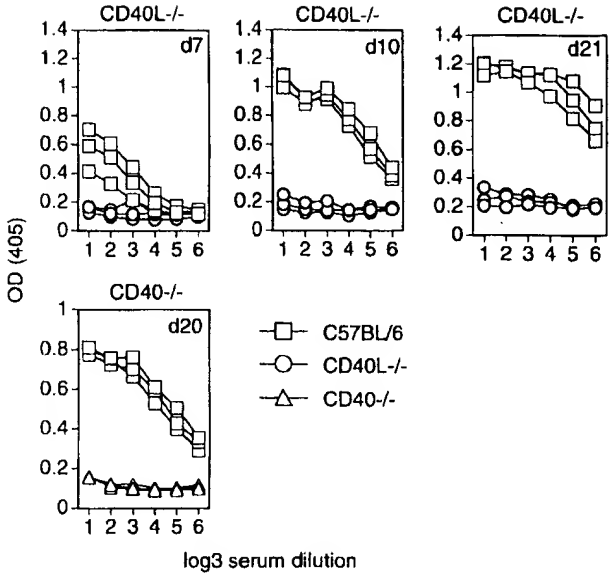

Figure 5. LCMV-NP specific IgG antibody response. CD40L-deficient (*circles*), CD40-deficient (*triangles*) and normal C56BL/6 (*squares*) mice were immunized with LCMV. LCMV-NP-specific IgG titers were determined 7, 10, and 21 d after infection for CD40L-deficient and normal C56BL/6 mice. LCMV-NP specific IgG titers in CD40-deficient and normal C57BL/6 mice were assessed 20 d after LCMV infection in 30-fold prediluted sera. Each line represents one individual mouse. Variations were smaller than two dilution steps. One of three comparable experiments is shown.

Th Cell Effector Function: Cognate Help to B Cells. It has already been demonstrared in detail that T-dependent IgG humoral immune responses are strictly dependent on a functional CD40-CD40L interaction and that a missing ligand in this complex totally abrogates germinal center formation as well as immunoglobulin isotype switch and generation of memory B cells (1–10). To ensure that Ig responses to LCMV as a model viral antigen require CD40-CD40L engagement, we infected CD40-, CD40L-deficient, or C57BL/6 mice with LCMV and then measured antiviral primary antibody responses. VSV was used as a second viral model antigen.

Infection of normal mice with LCMV leads to a pronounced IgG response specific for the nucleoprotein of LCMV (LCMV-NP) (Fig. 5). In contrast, no LCMV-NP-specific IgG titers were obtained using CD40L- or CD40-deficient mice (Fig. 5). Thus, CD40-CD40L interaction is crucial for isotype class switching in response to LCMV-infection.

In addition, we analyzed virus neutralizing antibody responses in CD40L-deficient and normal mice after infection with VSV. VSV infection of mice normally induces a type 1 T-independent neutralizing IgM-response followed by a T-dependent neutralizing IgG response (45–47). As expected control C57BL/6 mice mounted high neutralizing IgM and IgG responses (Fig. 6). In contrast, CD40L-deficient mice mounted a neutralizing T-independent IgM response but no VSV-specific IgG was detected upon VSV infection (Fig. 6). The same results were obtained using CD40-deficient mice (data not shown). These data corroborate previous findings in nonviral systems, demonstrating that cognate T-B cooperation leading to Ig class switching is critically dependent on functional CD40-CD40L interactions.

Th Cell Effector Function: Inflammatory Reaction. Infection of normal mice with LCMV in the hind footpads induces a local inflammatory reaction consisting of two sequential swelling phases. A pronounced swelling reaction mediated by $CD8^+$ T cells is observed 7–10 d after infection; this is followed by a $CD4^+$ T cell–mediated swelling phase declining around day 14–16 after infection (46, 48). The second phase of the swelling reaction exhibits no sharp peak but has the shape of a shoulder. We analyzed whether or not the second swelling phase mediated by LCMV-specific $CD4^+$ T cells was dependent on CD40-CD40L interactions. CD40-deficient mice as well as heterozygous littermates were infected with LCMV in both hind footpads and the subsequent swelling reaction was monitored daily (Fig. 7 *A*). In fact, there was a tendency noted that CD40-defi-

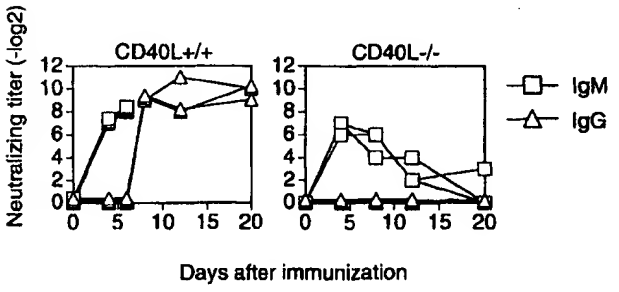

Figure 6. VSV neutralizing antibody response. CD40L-deficient and C57BL/6 mice were immunized with VSV. VSV-neutralizing IgM titers (*squares*) and VSV-neutralizing IgG titers (*triangles*) were determined from 40-fold prediluted sera 4, 6, 8, 12, and 20 d after infection. Each line represents one individual mouse. One of three comparable experiments is shown.

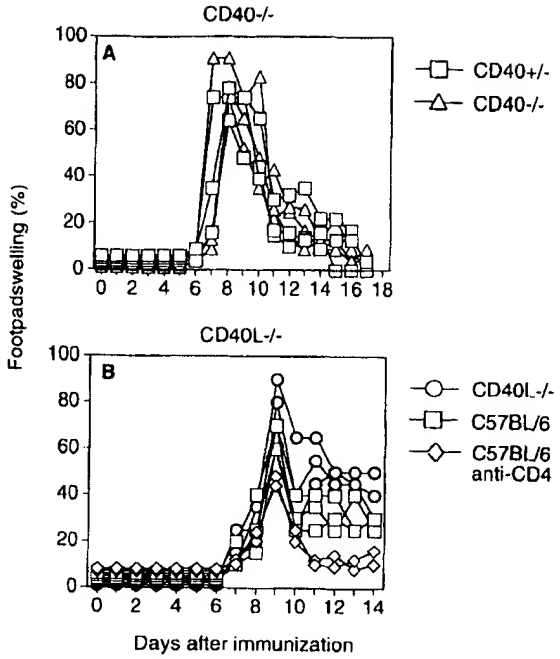

Figure 7. LCMV-specific foot pad swelling reaction. In *A* CD40-deficient mice (*triangles*) and heterozygous littermates (squares) were immunized into the footpads with 200 PFU of LCMV and the local LCMV-specific foot pad swelling reaction was daily monitored. In *B* LCMV-specific foot pad swelling reaction was similarly analyzed in CD40L-deficient (*circles*) and normal C57BL/6 mice (*squares*) as well as in anti-CD4 treated C57BL/6 mice (*diamonds*). Each line represents one individual mouse.

cient mice exhibited a slightly increased maximal early CD8-dependent peak response and that subsequently the CD4-dependent shoulder tended to be slightly higher. Thus, neither the $CD8^+$ T cell–mediated swelling phase nor in the $CD4^+$ T cell–mediated swelling phase was dependent upon a functional CD40 molecule. Compatible results were obtained with CD40L-deficient mice (Fig. 7 *B*). This indicates comparable abilities to mount LCMV-specific inflammatory reactions in the presence or absence of CD40-CD40L interaction. To confirm that the second phase of the swelling reaction was actually $CD4^+$ T cell mediated, C57BL/6 mice were treated with a $CD4^+$ T cell-depleting monoclonal antibody at day 6 and 8 after inoculation with LCMV. Foot pad swelling was less marked and the subsequent decline was much more rapid compared to untreated controls (Fig. 7 *B*).

Th Cell Effector Function: Antiviral Protection. A third Th cell effector function was analyzed in CD40L-deficient and normal mice. VSV primed C57BL/6 mice have been shown to rapidly control replication of a recombinant vaccinia virus expressing the glycoprotein of VSV ($VaccG_{IND}$). This antiviral protective mechanism is virtually exclusively mediated by IFNγ and TNFα which are secreted by VSV glycoprotein-specific $CD4^+$ T cells (37, 49). Neither $CD8^+$ T cells nor VSV-neutralizing antibodies have been shown to be protective in this infection. Because G_{IND} is not expressed in the $VaccG_{IND}$ envelope, VSV-specific antibodies cannot bind or neutralize $VaccG_{IND}$ recombinant virus. CD40L-deficient and normal mice were infected with VSV and 8 d later the VSV primed mice as well as naive control animals were challenged intraperitoneally by infection with $VaccG_{IND}$. Vaccinia titers in the ovaries were determined 5 d after challenge (Fig. 8). As with the foot pad swelling results (Fig. 7 *B*), we did not observe a reduced $CD4^+$ T cell mediated antiviral effector function in CD40L-deficient as compared to CD40L-competent mice.

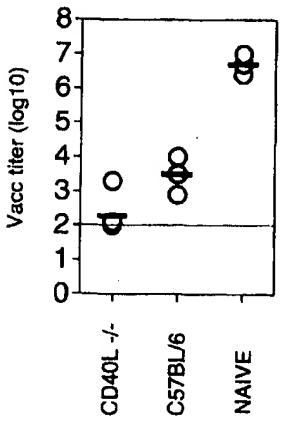

Figure 8. $CD4^+$ T cell dependent protection against challenge infection with recombinant vaccinia virus. CD40L-deficient and normal C57/BL6 mice were immunized with 2×10^6 PFU of VSV and 8 d later these primed mice as well as naive control mice were challenged i.p. with 5×10^6 PFU $VaccG_{IND}$. Vaccinia titers in ovaries were determined 5 d after challenge infection. One of two experiment is shown.

Discussion

Many reports have focussed on the key importance of the CD40-CD40L interaction for the generation of humoral immune responses (1–6). This was confirmed here for antiviral IgG immune responses. In contrast to most previous reports, this study examined in addition the role of CD40-CD40L interaction on Th cell activation, proliferation and effector functions. Surprisingly, neither Th cell induction nor Th cell effector functions other than cognate help for B cells were compromised by the lack of the CD40- or the CD40L molecules.

CD40 is expressed on different APCs such as dendritic cells and B cells and macrophages (19–23). In addition, it has been shown that CD40-CD40L interaction induces upregulation of MHC class II, B7 and ICAM-1 on dendritic cell lines which potentiate the stimulatory capacity of the APC (50). The CD40-CD40L interaction-dependent upregulation of these and maybe other accessory molecules on the surface of the APC signals then back to the T cell, a process which may be supported by interleukins in addition. Thus, it could be expected that activation and proliferation of specific Th cells would be impaired in the absence of the CD40-CD40L interaction as in fact has been recently reported (51, 52). However, we report here that antigen-specific Th cells are induced normally in vivo in the absence of a functional CD40-CD40L interaction after two different virus infections; hence the formation of this receptor-ligand pair is apparently not critical for the activation of naive virus-specific Th cells. Since B cell activation and proliferation seems to depend critically upon the presence of cognate T-help (5, 7, 53, 54), absence of CD40-CD40L interaction will interfere with proliferation of spe-

cific B cells and therefore reduce class II associated antigen presentation by specific B cells. Because it has been reported that antigen presentation by B cells is important for the normal induction of T-helper cells in several systems (38–44), our results suggest that either B cell proliferation and activation is impaired to a smaller degree than expected in the absence of CD40-CD40L interaction, or, alternatively and more likely, that the presence of specific B cells plays only a minor if any role for the induction of T-helper cells with the infectious virus-derived antigens studied here (55). This latter possibility is supported by the fact, that Th cells primed in the absence of B cells proliferated normally and could mediate isotype switch after adoptive transfer (Fig. 3, *C* and *D*). These findings are in contrast to recently presented data (51, 52). Grewal et al. (51) showed a significant impairment of KLH- or HEL-specific T cell priming in mice lacking CD40 ligand. This impairment was evidenced by strongly reduced recall proliferation responses and by a failure of adoptively transferred CD40L-deficient T cells to expand in vivo after antigenic challenge. In contrast to these findings, van Essen et al. observed normal KLH-specific Th cell induction in CD40-deficient mice, but described these KLH-primed T cells as being qualitatively different from T cells primed in a CD40-competent environment; they were apparently not able to provide help to B cells. These differences concerning the importance of a functional CD40-CD40L interaction for the induction and effector functions of T cells between the virus models used in this study and the soluble KLH antigen system used by Grewal et al. (51) and by van Essen et al. (52) probably reflect different critical in vivo mechanisms governing both the induction and effector functions of T cells. There are at least two possible explanations that might account for these differences: (*a*) The efficient activation of T cells after a viral infection such as with LCMV might over-ride some of the more subtle requirements for responses to soluble protein antigens such as KLH. In addition, the failure of CD40-deficient mice to resolve a Leishmania infection (H. Kikutani, personal communication), despite exhibiting a Leishmania-resistant genetic background, could be due to the lack of CD40 signals to macrophages or dendritic cells which then could lead to a deficit in IL-12 production and therefore to an impaired Th1 development. Additional evidence for a requirement of a functional CD40-CD40L interaction for IL-12 induction in vivo and hence the induction of a Th1 response has recently been presented by Stüber et al. (56). Thus, T helper cells may influence their responses via CD40L indirectly by activating their APCs. In a viral infection the direct secretion of IFN and possibly other interleukins by T cells may render this pathway less critical. (*b*) Alternatively, B cells may be of key importance as APCs for T cell induction in a soluble antigen system in contrast to virus model infections. There still exists an unresolved controversy on the issue whether B cells play a central role in the initiation of T cell immune responses. Even studies using the same experimental antigen such as KLH as soluble antigen and B cell-deficient mice revealed different results: Epstein et al. (57) reported successful T cell priming in B cell-deficient mice using KLH as antigen whereas Liu et al. (44) failed to obtain T cell priming in B cell-deficient mice using either KLH or OVA as soluble protein antigens. Constant et al. (58) demonstrated that mice lacking B cells were impaired in their priming of T cells to protein but not to peptide antigens. This dominant role of B cells as APCs found in several experimental systems using soluble antigens is possibly abrogated if the CD40 signaling pathway is not functional. We deliberately chose an experimental system where T cell induction is not dependent on B cells functioning as APCs and analyzed the role of CD40-CD40L interaction in T cell priming and Th cell effector functions independently of the presence or absence of B cells.

The effector functions of Th cells are differentially influenced by CD40-CD40L interaction. Thymus-dependent Ig-isotype switched humoral immune responses are completely abrogated in CD40- or CD40L-deficient mice whereas thymus-independent IgM-responses to VSV are comparable to normal mice; the latter apparently do not require a functional interaction between CD40 and its ligand. Although T-helper cells do not induce antibody isotype switching in B cells in the absence of CD40-CD40L interaction, $CD4^+$ T cells could be functionally primed in CD40-deficient mice since upon transfer they were able to provide help for CD40-competent B cells to undergo isotype switch. These findings are in contrast to the recent data of van Essen et al. (52) showing that KLH-specific T cells primed in a CD40-deficient environment are not able to help CD40-competent B cells. Although the experimental setups are not identical, it can be concluded that Th cell priming by virus infection seems to be considerably more efficient in a CD40-deficient environment than is possible for soluble protein antigens. Not only were induction, proliferation and cytokine production of specific T-helper cells normal in the absence of CD40-CD40L interaction but also effector functions of T-helper cells other than mediating Ig isotype switching were largely normal in the absence of CD40 or CD40L. $CD4^+$ T cell mediated inflammatory reaction upon LCMV infection was not impaired in CD40- or CD40L-deficient mice. In addition, Th cell-dependent antiviral protection was not impaired by the lack of CD40-CD40L interaction: activated VSV-specific Th cells in CD40L-deficient mice were able to inhibit the replication of VSV-G recombinant vaccinia virus by a cytokine mediated effector function; this protective Th cell function has been shown to be mediated by release of IFNγ and TNFα by activated $CD4^+$ Th cells (37). It has recently been suggested, that CD40L may exert a direct protective effect against vaccinia virus (59). Our data show that Th cells can efficiently protect against vaccinia virus replication also in the absence of CD40L, suggesting that this parameter is not limiting under the conditions tested in vivo.

In conclusion, these results reveal a dichotomy in the role of the CD40-CD40L interaction in Th cell effector functions: the functional interaction is of critical importance in T-B cell cooperation for the induction of thymus-dependent humoral immune responses but the same inter-

action is not limiting for Th cell mediated effector functions such as virus-induced inflammatory reactions or antiviral protection, which probably depend on interactions between Th cells with macrophages and/or dendritic cells. Our data strongly suggest that CD40-CD40L interaction divides anti-viral Th cell effector functions in two categories: one category consisting of cognate T help mediating B cell activation followed by Ig class switching which is strictly dependent on a functional CD40-CD40L interaction; and a second category, represented in this report by virus-induced, $CD4^+$ T cell-mediated inflammatory reaction as well as antiviral protection is largely independent of a functional CD40-CD40L interaction. Similarly, activation and proliferation of Th cells, albeit not a Th cell effector function, may be placed in this second category. Thus, CD40-CD40L interaction is critically and probably unidirectionally-needed for B cell activation followed by Ig class switch, but in addition is possibly of importance for macrophage/dendritic cell activation especially in the case of nonviral antigens.

We thank Werner Müller and Klaus Rajewsky for generously providing us with the IgM-deficient mice. We thank Awen Gallimore for helpful discussions, Alana Althage for excellent technical help, and Yvonne Deflorin for secretarial assistance.

This work was supported by grants from the Swiss National Science Foundation (21-32179.91) and the Kanton of Zürich, Switzerland and the Human Science Frontier Program.

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Received for publication 9 November 1995 and in revised form 26 February 1996.

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Brief Definitive Report

B Lymphocytes Are Essential for the Initiation of T Cell–mediated Autoimmune Diabetes: Analysis of a New "Speed Congenic" Stock of NOD.*Ig*μ^{null} Mice

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Summary

The T lymphocytes mediating autoimmune destruction of pancreatic β cells in the nonobese diabetic (NOD) mouse model of insulin-dependent diabetes mellitus (IDDM) may be generated due to functional defects in hematopoietically derived antigen-presenting cells (APC). However, it has not been clear which particular subpopulations of APC (B lymphocytes, macrophages, and dendritic cells) contribute to the development and activation of diabetogenic T cells in NOD mice. In the current study we utilized a functionally inactivated immunoglobulin (*Ig*)μ allele (*Igμ*^{null}) to generate a "speed congenic" stock of B lymphocyte–deficient NOD mice that are fixed for linkage markers delineating previously identified diabetes susceptibility (*Idd*) genes. These B lymphocyte NOD.*Igμ*^{null} mice had normal numbers of T cells but were free of overt IDDM and insulitis resistant, while the frequency of disease in the B lymphocyte intact segregants was equivalent to that of standard NOD mice in our colony. Thus, B lymphocytes play a heretofore unrecognized role that is essential for the initial development and/or activation of β cell autoreactive T cells in NOD mice.

Insulin-dependent diabetes mellitus (IDDM) in the nonobese diabetic (NOD) mouse model results from autoimmune destruction of pancreatic β cells mediated by both CD4$^+$ and CD8$^+$ T lymphocytes (1, 2). However, while T lymphocytes are clearly the final mediators of β cell destruction in NOD mice, there is evidence that these autoreactive effectors are generated as a consequence of functional defects in hematopoietically derived APC such as B lymphocytes, macrophages, and dendritic cells (2). Diabetes susceptibility (*Idd*) genes both inside and outside of the unusual *H2*^{g7} MHC haplotype appear to contribute to diabetogenic APC defects in NOD mice. However, the mechanisms whereby APC contribute to the development of autoimmune IDDM in NOD mice are unknown. Possibilities include a unique ability of NOD APC to process and immunologically present certain β cell proteins, and/or a reduced capacity to activate tolerogenic mechanisms that normally anergize or delete diabetogenic T lymphocytes. Another, not mutally exclusive, possibility is that NOD APC preferentially induce β cell autoreactive CD4$^+$ T lymphocytes to secrete cytokines characteristic of a potentially pathogenic Th1 response, rather than those of a Th2 response that may dampen pathogenesis. However, it is not known if any of these potentially diabetogenic dysfunctions in NOD mice are exerted by all or particular subpopulations of APC.

It has been proposed that B lymphocytes may play a more critical role in the induction of immunological tolerance than other APC populations. This possibility is supported by reports that B lymphocytes have a greater capacity than other types of APC to induce T cell anergy and to preferentially activate Th2 rather than Th1 cytokine responses (3, 4). However, this conclusion was questioned by a recent report (5) demonstrating that peripheral T cell tolerance could be induced normally in mice made B lymphocyte deficient by congenic transfer of the previously described Igμ allele (6) that has been functionally disrupted by homologous recombination (originally abbreviated μMT^0, now formally designated *Igh6*^{tm1Cgn}, and here designated *Igμ*^{null} for clarity). Specific subpopulations of APC may also differentially contribute to IDDM susceptibility in NOD mice if they present varying mosaics of β cell autoantigens to T lymphocytes. Indeed, some proteins can only be antigenically processed and presented to T cells by B lymphocytes (7). This process may lead to the development of autoimmunity if B lymphocytes activate normally quiescent self-reactive T cells that had escaped tolerogenic mechanisms because the antigenic determinants they recognize are not

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/11/2049/05 $2.00
Volume 184 November 1996 2049–2053

normally processed and presented by other populations of APC (8). To understand the relative roles various APC subpopulations play in the development of autoimmune IDDM, we examined if pathogenesis was altered in a congenic stock of B lymphocyte–deficient NOD.$Ig\mu^{null}$ mice.

Materials and Methods

Development of a "Speed Congenic" Stock of NOD.$Ig\mu^{null}$ Mice. The $Ig\mu^{null}$ allele functionally disrupted by insertion of a neomycin resistance gene (*neo*^{r}) was backcrossed from the original chimeric stock with a mixed 129 and C57BL/6 (B6) genome (6) onto the NOD/Lt inbred background. The heterozygous carriers of the $Ig\mu^{null}$ allele used as breeders at each backcross generation were identified by typing DNA isolated from PBL by PCR with the primer set 5′-GCTATTCGGCTATGACTGGG-3′ and 5′-GAAGGCGATAGAAGGCGATG-3′, which generates a 706-bp product from within the *neo*^{r} insert. Each of 35 amplification cycles on a PTC-100 thermal cycler (MJ Research, Watertown, MA) consisted of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s. Using previously described PCR methodologies (9, 10), segregants from the fourth and sixth backcross (N5 and N7, respectively) generation were genotyped for the microsatellite markers shown in Table 1 that are linked to the indicated *Idd* loci. At the N7 backcross generation, $Ig\mu^{null/+}$ heterozygotes shown by these PCR analyses to be fixed as homozygous for NOD alleles at the indicated linkage markers of *Idd* susceptibility loci were intercrossed. PBL from the resulting F1 progeny were typed by two-color flow cytometric analysis (FACScan®, Becton Dickinson and Co., San Jose, CA) for the presence or absence of B lymphocytes using an FITC-conjugated goat polyclonal antiserum specific for mouse IgM (Southern Biotechnology Associates, Birmingham, AL) and the B220 specific mAb RA3-6B2 conjugated to PE. Segregants lacking B lymphocytes were scored as NOD.$Ig\mu^{null}$ homozygotes, and have been maintained by brother–sister mating. B lymphocyte intact N7F1 progeny were scored as wild-type $Ig\mu^{+/+}$ homozygotes if the *neo*^{r} insert could not be detected by PCR. N7F1 progeny carrying the *neo*^{r} insert, but positive for B lymphocytes, were scored as $Ig\mu^{null/+}$ heterozygotes. All mice were maintained under specific pathogen–free (SPF) conditions and allowed free access to food (Agway diet NIH 31A; PMI Feeds Co., South Henley, MO) and acidified drinking water.

Assessment of Diabetes, Insulitis, and Leukocyte Subsets. B lymphocyte intact and deficient segregants were monitored weekly for the development of glycosuria with Ames Diastix® (kindly supplied by Miles Laboratories Inc., Elkhart, IN). Glycosuric values of ≥3 were considered diagnostic of diabetes onset. Pancreases from mice that remained normoglycemic through 20 wk of age were fixed in Bouin's solution, sectioned at three nonoverlapping levels, and stained with aldehyde fuchsin for histological analysis of insulitis development. Islets (at least 25/mouse) were individually scored as follows: 0, no lesions; 1, perinsular leukocytic aggregates, usually periductal infiltrates; 2, <25% islet destruction; 3, >25% islet destruction; 4, complete islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are presented as mean insulitis score ± SEM for the indicated number of B lymphocyte intact or deficient segregants. Splenic leukocytes from mice that remained non diabetic through 20 wk of age were typed by FACS® for the presence of B lymphocytes with a goat polyclonal antiserum specific for mouse Ig (Southern Biotechnology Associates), and for macrophages (MØ), CD4$^+$, and CD8$^+$ T lymphocytes with the mAbs M1/70, GK1.5, and 53-6.72, respectively.

Table 1. *Linkage Markers Analyzed to Fix NOD-derived Idd Loci to Homozgosity in the NOD.$Ig\mu^{null}$ Congenic Stock*

Idd locus/chromosome	Linkage marker homozygous for NOD allele	Relative microsatellite size
Idd1=H2^{g7}/17	**D17Mit34=C4*	B6=129>NOD
Idd2/9	**D9Mit25*	NOD>B6>129
Idd3/3	*D3Mit206*	NOD>129>B6
	**D3Nds6=Il2*	B6>NOD=129
	D3Mit95	NOD>B6=129
Idd4/11	*D11Mit115*	NOD>B6=129
	**D11Nds16=Acrb*	NOD=129>B6
	D11Mit320	129>NOD>B6
Idd5/1	**D1Mit5*	NOD=129>B6
	D1Mit18	NOD>B6=129
	**D1Mit46*	NOD=129>B6
Idd6/6	*D6Mit52*	B6=129>NOD
	D6Mit339	B6>NOD>129
Idd7/7	**D7Mit20*	B6=129>NOD
Idd8, Idd12/14	**D14Mit11*	B6>NOD
	D14Mit110	129>NOD> B6
	D14Mit222	NOD>B6=129
Idd9, Idd11/4	**D4Mit59*	NOD>B6=129
Idd10/3	*D3Nds11*	B6=129>NOD
	**D3Nds8=Tshb*	NOD=129>B6
	D3Mit103	B6>NOD>129
Idd13/2	*D2Mit395*	129>B6>NOD
	**B2m*	NOD=129≠B6‡
	D2Mit17	NOD>129>B6
Idd14/13	**D13Mit61*	NOD>B6=129
Idd15/5	**D5Mit48=Pgyl*	B6>NOD
	D5Mit69	NOD>B6=129

Microsatellites markers with the indicated allelic size variants were typed in N5 backcross mice and in the N7 segregants used for the intercross.

*Markers typed as homozygous for allelic variants characteristic of NOD in the single N5 female serving as the progenitor for all mice in subsequent backcross generations.

‡β2m allelic variants were typed as previously described (9) by BglI digestion of the PCR product.

Results

Genetic Characterization of the NOD.$Ig\mu^{null}$ "Speed Congenic" Stock. Heterozygous carriers of the $Ig\mu^{null}$ allele from the N5 backcross generation were genotyped by PCR for the markers denoted by an asterisk in Table 1 that were previously shown to be closely linked to the indicated *Idd* loci.

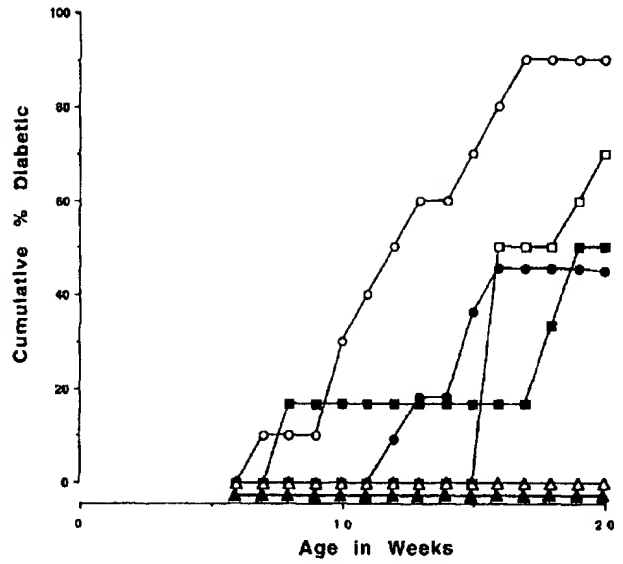

Figure 1. Diabetes development through 20 wk of age in NOD.$Ig\mu^{null}$, NOD.$Ig\mu^{null/+}$, and NOD.$Ig\mu^{+/+}$ segregants at the BC6 generation. Symbols: NOD.$Ig\mu^{null}$ females (n = 8, △) and males (n = 9, ▲); NOD.$Ig\mu^{null/+}$ females (n = 10, ○) and males (n = 11, ●); NOD.$Ig\mu^{+/+}$ females (n = 10, □) and males (n = 6, ■). Chi-squared analysis demonstrated that while significantly higher ($P < 0.01$) than in B lymphocyte–deficient NOD.$Ig\mu^{null}$ male and females, the cumulative incidence of IDDM at 20 wk of age did not differ statistically ($P > 0.9$) between sex-matched B lymphocyte–intact $Ig\mu^{null/+}$ or $Ig\mu^{+/+}$ segregants.

This analysis identified a single N5 female that was homozygous for allelic variants characteristic of NOD mice at all of these *Idd* linkage markers. This female served as the progenitor for all mice in subsequent backcross generations. We also genotyped the NOD, 129, and B6 parental strains for a series of additional microsatellites marking chromosomal regions carrying *Idd1-15*. This enabled us to identify the series of linkage markers shown in Table 1 that delineate NOD genomic elements from those derived from either the 129 or B6 strains. Typing of these markers confirmed the homozygous presence of NOD derived genome at all previously identified *Idd* loci in the N7 progenitors used for the intercross (Table 1).

NOD.$Ig\mu^{null}$ Mice Are IDDM and Insulitis-resistant. Currently, IDDM develops in 90% of standard NOD/Lt female and 63% of male mice at The Jackson Laboratory by 1 year of age. This susceptibility has been fully reconstituted in the speed congenic stock of NOD.$Ig\mu^{null}$ mice as evidenced by the IDDM incidence in the B lymphocyte positive segregants from the N7 backcross generation. By 20 wk of age, IDDM developed in 70% (7/10) of female and 50% (3/6) of male N7F1 NOD.$Ig\mu^{+/+}$ segregants. Similarly, IDDM also developed in 90% (9/10) of female and 45.5% (5/11) of male N7F1 NOD.$Ig\mu^{null/+}$ segregants. Mean insulitis scores of the B lymphocyte intact segregants that remained free of overt IDDM through 20 wk were 3.89 ± 0.10 (n = 4) in females and 3.28 ± 0.32 (n = 9) in males. The extensive insulitis and high frequency of IDDM in these two groups of B lymphocyte intact N7F1 segregants is equivalent to that observed in our standard NOD mice at the same age. This provided a functional confirmation of the linkage marker analyses (Table 1) demonstrating that the NOD-derived *Idd* loci necessary for disease development had been fixed in the N7 segregants. In marked contrast, IDDM failed to develop in any B lymphocyte–deficient female (0/8) or male (0/9) N7 NOD.$Ig\mu^{null}$ mice (Fig. 1). These NOD.$Ig\mu^{null}$ mice were also virtually free of insulitis as evidenced by mean insulitis scores at 20 wk of age of 0.31 ± 0.06 (n = 5) in females and 0.23 ± 0.02 (n = 5) in males. These scores reflected, at most, the presence of perivascular/periductal leukocytic aggregates, but no intraislet infiltrates. This virtual absence of insulitis in NOD.$Ig\mu^{null}$ mice indicates that IDDM development is not merely delayed, but that β cell–autoreactive T cell responses are not initiated in this stock. The $Ig\mu$ structural gene is located on distal chromosome 12 in a region where genome-wide scans have failed to detect any *Idd* loci. Collectively, these results indicate that elimination of B lym-

Table 2. *Splenic Leukocyte Profiles of NOD.$Ig\mu^{null}$, NOD.$Igm\mu^{null}$1, and NOD.$Ig\mu^{+/+}$ Segregants*

Genotype	Percent B cells	No. B cells (× 10^6)	Percent CD4 T cells	No. CD4 T cells (× 10^6)	Percent CD8 T cells	No. CD8 T cells (× 10^6)	Percent MØ	No. MØ (× 10^6)
$Ig\mu^{null}$	0.12	0.05	58.1	22.6	21.8	8.0	8.6	2.9
(n = 3)	± 0.05	± 0.03	± 3.6	± 8.2	± 0.7	± 2.6	± 1.4	± 0.8
$Ig\mu^{null/+}$	35.8	34.3	32.4	30.7	8.5	8.1	5.9	5.5
(n = 4)	± 1.2	± 3.0	± 1.1	± 2.4	± 1.2	± 1.4	± 0.6	± 0.5
$Ig\mu^{+/+}$	39.9	40.6	28.8	29.4	9.1	9.3	5.7	5.6
(n = 6)	± 1.2	± 4.1	± 1.0	± 3.1	± 0.7	± 1.1	± 0.5	± 0.4

Splenic leukocytes from BC6 segregants remaining nondiabetic through 20 wk of age were typed by FACS® as described in Materials and Methods for the presence of B lymphocytes, CD4 and CD8 T lymphocytes, and MØ. Data represent percentage or absolute number ± SEM of each leukocyte population.

phocytes by congenic transfer of the $Ig\mu^{null}$ mutation is sufficient to block IDDM development in an otherwise genetically susceptible NOD mouse stock.

Normal T Cell Lymphocyte Development in B Lymphocyte–deficient NOD.$Ig\mu^{null}$ Mice. It was important to determine if the absence of IDDM and insulitis in NOD.$Ig\mu^{null}$ mice resulted solely from the elimination of B lymphocytes, or whether this mutation also rendered mice T lymphopenic. Thus, we used FACS® analysis to compare the percentages and absolute numbers of B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes, and MØ in the spleens of 20-wk-old NOD.$Ig\mu^{null}$, NOD.$Ig\mu^{null/+}$, and NOD.$Ig\mu^{+/+}$ segregants. As shown in Table 2, B lymphocytes were present and did not differ in proportion or absolute number in spleens of $Ig\mu^{null/+}$ and $Ig\mu^{+/+}$ segregants. As expected, B lymphocytes were absent in spleens of $Ig\mu^{null}$ segregants. The proportion of $CD4^+$ and $CD8^+$ T lymphocytes in spleens of $Ig\mu^{null}$ segregants was two to three times higher than in $Ig\mu^{null/+}$ and $Ig\mu^{+/+}$ segregants. However, this was due solely to the absence of B lymphocytes in mice homozygous for the $Ig\mu^{null}$ allele, since the absolute numbers of splenic $CD4^+$ and $CD8^+$ T lymphocytes were similar in all three segregation classes. Similarly, the proportion, but not the absolute number of MØ was also increased in spleens of $Ig\mu^{null}$ segregants compared with either class of B lymphocyte–intact segregants. When compared at 6 wk of age, total numbers of splenic T cells and MØ also did not differ between NOD.$Ig\mu^{null}$ and standard NOD mice. Thus, the genetic elimination of B lymphocytes does not appear to quantitatively effect the development of other leukocyte populations in NOD mice.

Discussion

It has been previously demonstrated that T lymphocytes from diabetic NOD donors can efficiently transfer disease to neonatal recipients depleted of B lymphocytes by treatment with a μ-specific mAb (11). Thus, after diabetogenic T lymphocytes have been generated they can clearly mediate destruction of pancreatic β cells in the absence of B lymphocytes. However, our current study demonstrates that neither IDDM nor significant levels of insulitis develop in B lymphocyte–deficient NOD.$Ig\mu^{null}$ mice that have fixed the necessary NOD-derived *Idd* susceptibility loci to permit disease development in the presence of B lymphocytes.

Verifying that all *Idd* loci normally necessary for IDDM development are fixed in an NOD congenic stock is an essential prerequisite when determining whether the genetic elimination of a particular immunological component affects pathogenesis. This is illustrated by two consecutive studies from the same group, which analyzed the development of β cell autoimmunity in NOD mice congenic for a segment of chromosome 14 derived from the 129 strain that contained a functionally inactivated TCR-α locus (12, 13). It was originally reported that in a heterozygous state, the inactivated TCR-α locus could block the development of β cell autoimmunity in NOD mice (12). However, as portions of the congenic segment recombined to NOD type with further backcrossing, it was subsequently found that the originally reported resistance was not due to the inactivated TCR-α locus, but rather to the presence of linked 129-derived resistance alleles at the previously identified *Idd8* and/or *Idd12* loci on chromosome 14 (13). Our genotypic analysis, coupled with the normal rate of disease development in the B lymphocyte–intact segregants, indicates that complete IDDM resistance in the NOD.$Ig\mu^{null}$ stock cannot be ascribed to the presence of 129 or B6 derived resistance alleles at any previously identified *Idd* loci. To date, when any single non-MHC *Idd* resistance locus has been congenically fixed to homozygosity on the NOD background, it has not resulted in complete IDDM and insulitis resistance in females as observed in our NOD.$Ig\mu^{null}$ stock. Thus, it is highly unlikely that the absence of IDDM and insulitis in our NOD.$Ig\mu^{null}$ stock is due to a previously unidentified recessively acting 129 derived resistance allele linked to the functionally inactivated $Ig\mu$ gene. This conclusion is further supported by the fact that while numerous *Idd* susceptibility and resistance loci with major to very minor effects have mapped in outcross studies of NOD mice with a number of different diabetes resistant strains, none of these have mapped to chromosome 12 where $Ig\mu$ is located (1, 14). Hence, these past studies coupled with our current findings clearly indicate that B lymphocytes play a heretofore unrecognized role that is essential for the initial development and/or activation of β cell autoreactive T cells in NOD mice.

The mechanism(s) by which B lymphocytes contribute to the initial development of diabetogenic T lymphocytes in NOD mice remains to be elucidated. It has been previously proposed that in very young NOD mice, autoantibodies secreted by B lymphocytes bind to pancreatic β cells, and that this triggers an autoreactive T cell cascade through an Ab-dependent cell-mediated cytotoxicity (ADCC) response (15). However, if such an ADCC mechanism is essential to diabetogenesis in NOD mice, it cannot be mediated by maternally transmitted autoantibodies, since equivalent degrees of disease resistance were observed in NOD.$Ig\mu^{null}$ segregants derived from either B lymphocyte intact ($Ig\mu^{null/+}$) or deficient ($Ig\mu^{null}$) dams. Future studies will examine the possibility that diabetogenic T lymphocytes are initially triggered by an ADCC response by determining if disease resistance is abrogated in NOD.$Ig\mu^{null}$ mice injected with Ig isolated from standard NOD donors with high islet cell autoantibody titers. Another, not mutually exclusive, possibility is that B lymphocytes contribute to the development of autoimmune IDDM in NOD mice through their role as APC. It has been reported that B lymphocytes may have a greater capacity than other APC to activate immunoregulatory functions that may block the development of IDDM, such as rendering autoreactive T cells anergic or skewing the pattern of cytokines they produce from a Th1 to a Th2 profile (3, 4). However, our finding that autoimmune IDDM is completely inhibited rather than enhanced in NOD.$Ig\mu^{null}$ mice indicates that the development and/or activation of β cell–autoreactive T cells is not normally blocked by such B lymphocyte–controlled immunoregulatory mechanisms.

In this way our results are in agreement with a recent report that B lymphocytes are not required for the induction of peripheral T cell tolerance (5). On the other hand, our data do not exclude the possibility that the diabetogenic function of B lymphocytes in NOD mice is as APC with a unique ability to process and present certain β cell autoantigens to autoreactive T cells. This possibility is currently being tested by determining if the B lymphocyte–deficient NOD.$Ig\mu^{null}$ stock differs from standard NOD mice in ability to process and present certain candidate β cell autoantigens such as glutamic acid decarboxylase and insulin to T cells (16–18).

Most previous analyses of B lymphocyte function in the NOD mouse have focused on the spontaneous development of autoantibodies against putative islet cell antigens. Autoantibody production in NOD mice has been assumed to be a secondary consequence of β cell destruction, and hence, B lymphocytes have been regarded as accessories rather than primary instigators of pathogenesis. Our results clearly delineate a previously unidentified primary pathogenic role for B lymphocytes in IDDM initiation in the NOD mouse. The mechanism(s) by which B lymphocytes contribute to the initiation of autoimmune IDDM in NOD mice remain to be elucidated. However, knowledge of these mechanisms may provide new avenues for predicting and/or preventing the development of autoimmune IDDM in humans.

This work was supported by National Institutes of Health grants DK46266 and DK51090 (D.V. Serreze), DK36175 and DK27722 (E.H. Leiter), CA20408 and AI30389 (L.D. Shultz), as well as by grants from the Juvenile Diabetes Foundation International, and Cancer Center Support CA34196.

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Received for publication 29 July 1996 and in revised form 28 August 1996.

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International Immunology, Vol. 9, No. 8, pp. 1159–1164

Direct evidence for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice

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Keywords: autoimmunity, B lymphocytes, diabetes, knockout, rodent

Abstract

The non-obese diabetic (NOD) mouse is an excellent animal model of autoimmune diabetes associated with insulitis. The progression of insulitis causes the destruction of pancreatic β cells, resulting in the development of hyperglycemia. Although it has been well documented that T cells are required for the development of insulitis and diabetes in NOD mice, the importance of B cells remains unclear. To clarify the role of B cells in the pathogenesis of NOD mice, we therefore generated B cell-deficient NOD (B^-NOD) mice. Surprisingly, none (of 13) of the B^-NOD mice developed diabetes by 40 weeks of age, while the control littermates with B cells (B^+NOD) suffered from a high proportion (43 of 49) of diabetes. The insulin reactivity of B^+NOD mice was significantly impaired, while the B^-NOD mice showed a good insulin response, thus suggesting the pancreatic β cell function to be well preserved in B^-NOD mice. Although B^-NOD mice did develop insulitis, the extent of insulitis was significantly suppressed. These data thus provide the direct evidence that B cells are essential for the progression of insulitis and the development of diabetes in NOD mice.

Introduction

The non-obese diabetic (NOD) mouse develops diabetes concomitant with the progression of insulitis, thus providing an excellent animal model of human autoimmune diabetes mellitus (1–3). It has been proven that T cells are required for the development of insulitis and diabetes in NOD mice (3–15). Insulitis and diabetes did not develop in congenitally athymic nude mice and neonatally thymectomized NOD mice (4,5). Immunohistological studies revealed the predominant accumulation of T cells in the insulitic lesion (6,7). Treatment of mice with anti-T cell antibody prevented the development of the disease (8–10). In addition, adoptively transferred T cells could successfully induce insulitis and diabetes in irradiated young and newborn NOD mice (11–14).

Although these previous studies revealed the significant role of T cells, the role of B cells in the development of insulitis and diabetes remains obscure. B cells were not required for the transfer of diabetes (15), while immunohistochemical studies of insulitis revealed the accumulation of B cells in the later stage of insulitis (6,7). In addition, production of autoantibodies to pancreatic β cells is predictive of the later development of diabetes (16,17). These data raise the possibility that B cells may also play some role in the pathogenesis of insulitis and diabetes in NOD mice.

In the present study, in order to clarify the involvement of B cells in the development of insulitis and diabetes in NOD mice, we have generated B cell-deficient NOD mice by

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Transmitting editor: I. L. Weissman

Received 17 March 1997, *accepted* 28 April 1997

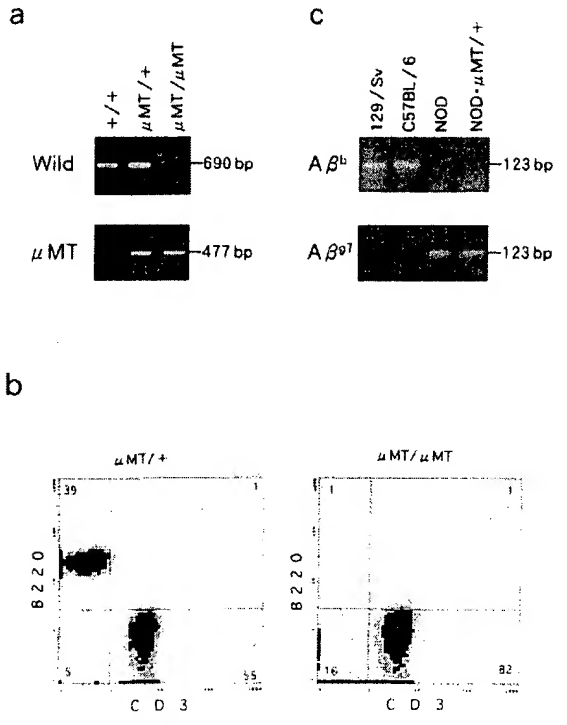

Fig. 1. Establishment of B cell-deficient NOD mice. μMT/+ B6 mice were backcrossed to +/+ NOD mice 8 times and μMT/+ NOD mice were produced. μMT/μMT NOD mice were obtained by crossing μMT/+ mice. (a) Homozygocity of the μMT allele was determined in a μMT/μMT NOD mouse. (b) Flow cytometric analysis revealed the absence of $B220^+$ B cells in peripheral blood mononuclear cells from a μMT/μMT NOD mouse. (c) The I-A_β gene of the class II molecules in the parental μMT/+ NOD mouse was I-A_β^7 as assessed by PCR.

backcrossing mice with the null μMT mutation to NOD mice. In the B cell-deficient NOD mice, diabetes did not develop over a 40-week observation period and the progression of insulitis was significantly suppressed, indicating the importance of B cells in the pathogenesis of insulitis and diabetes in NOD mice.

Methods

PCR to detect μMT and I-A_β^7 genes

The μMT allele was detected by PCR using genomic DNAs from tails. We used primer sets P-1988 (5′-TACAGCTCAGCTGTCTGTGG-3′) and P-neo^r (5′-TCTATCGCCTTCTTGACGAG-3′) to amplify the 477 bp products from the μMT allele where the neo^r gene was inserted in the membrane exon of the μ chain. Primer sets P-3542 (5′-CTGTCTTGCTTGCTCTGCTC-3′) and P-1988 were used to amplify 690 bp products from the wild allele. The PCR was performed at 95°C for 60 s, 65°C for 60 s and 72°C for 90 s, for 40 cycles on a DNA thermal cycler. PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. I-A_β genes of the class II molecules were determined as described previously (18). Genomic DNA (1 μg) was used for PCR by primers with additional *Eco*RI sites specific for I-A_β^b (5′-A_β^b: 5′-TCTAGAATTCACAGCGACATGGGCGAGC-3′ and 3′-A_β^b: 5′-TCTAGAATTCCGTAGTTGTGTCTGCACA-3′) and I-A_β^{g7} (5′-I-A_β^{g7}:5′-TCTAGAATTCACAGCGACGTGGGCGAGT-3′ and 3′-A_β^{g7}: 5′-TCTAGAATTCCGTAGTTGTGTCTGCACG-3′). I-A_β genes were amplified as follows: denaturing at 94°C for 1.5 min, annealing at 55°C for 1.0 min and extension at 72°C for 1.0 min, for 25 cycles on a DNA thermal cycler.

Flow cytometric analysis

The peripheral blood mononuclear cells of NOD mice were stained with phycoerythrin-labeled anti-B220 (Dainippon, Osaka, Japan), FITC-labeled anti-B220 (PharMingen, San Diego, CA) and biotinylated anti-CD3ε (PharMingen) mAb. The cells were washed with PBS containing 2% fetal bovine serum and 0.1% NaN_3, followed by streptavidin-Red670 (Gibco, Grand Island, NY) staining. The cells were then fixed with 1% paraformaldehyde and analyzed by a flow cytometer.

Assessment of serum Ig levels

Serum Ig levels were determined by ELISA. Briefly, sera for the Ig assay were collected from mice at 30 weeks of age. Plastic plates coated with anti-Ig (Rougier Biotech, Canada) were used after blocking with 5% non-fat milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0). After washing the plate with TBST, diluted serum samples were added to the plates and incubated at 37°C for 2 h. The bound IgM or IgG was detected with horseradish peroxidase-labeled goat anti-mouse IgM antibody (Tago, Burlingame, CA) or goat anti-mouse IgG antibody (Zymed, San Francisco, CA), and developed with TMB with the TMB microwell peroxidase substrate system (Kirkegaad & Perry, Gaithersburg, MD).

Glucose tolerance test and assay for serum insulin levels

Mice at 40 weeks of age were i.p. injected with 1 g/kg body weight of glucose and blood samples were then obtained at 0, 60 and 120 min after injection. The concentrations of insulin in the samples were determined by ELISA (Morinaga Biochem., Yokohama, Japan). All data are the means ± SEM, $n = 3$ per group.

Histological analysis

Blocks of pancreas were obtained at 12 and 30 weeks of age. For light microscopy, the pancreases were fixed with formalin and embedded in paraffin. Then, 4 μm thick sections made at 15 μm intervals for each pancreas were stained with hematoxylin & eosin. All islets were evaluated and the insulitis scores were determined as follows: 0: no mononuclear cell infiltration; 1: mild peri-insular mononuclear cell infiltration; 2: moderate mononuclear cell infiltration into the islets (granulation of <50%); 3: severe massive cell infiltration (granulation of >50%).

Results

Generation of B cell-deficient NOD mice

B cell-deficient (μMT/μMT) [129/Sv×C57BL/6 (B6)] mice were originally produced by disrupting the membrane exon of the IgM gene by gene targeting (19). μMT/+ B6 mice were

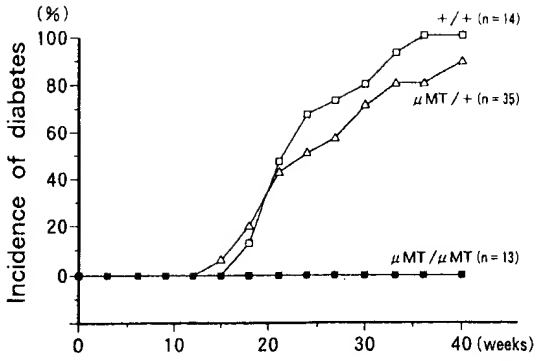

Fig. 2. Cumulative incidence of diabetes in the B⁻NOD and control B⁺NOD mice. Thirteen B⁻NOD (μMT/μMT) mice and control B⁺NOD littermates (15 +/+ and 34 μMT/+) were monitored for blood glucose levels for 40 weeks and the cumulative incidence of diabetes was determined. Open squares, +/+ B⁺NOD mice; open triangles, μMT/+ B⁺NOD mice; closed squares, μMT/μMT B⁻NOD mice.

previously generated by six backcrosses to B6 mice. μMT/+ B6 mice were backcrossed to +/+ NOD mice 8 times and μMT/+ mice with 99.6% NOD background were obtained. μMT/+ B⁺NOD males and females were crossed and assessed for their genetic background and also for the presence of B cell deficiency.

Thirteen μMT/μMT B⁻NOD mice and control B⁺NOD littermates (15 +/+ and 34 μMT/+) were obtained. The B⁻NOD and B⁺NOD mice were bred under specific pathogen-free conditions at the Kyushu University Animal Center. The mice were genotyped for μMT and wild-type alleles by PCR (Fig. 1a). Flow cytometric analysis of peripheral blood mononuclear cells of B⁻NOD mice revealed the absence of B220⁺ B cells (Fig. 1b). The levels of serum IgG and IgM in B⁺NOD mice were 12.50 ± 3.73 and 0.80 ± 0.05 g/l respectively, while those of B⁻NOD mice were <0.4 g/l for IgG and <0.02 g/l for IgM, thus confirming the lack of Ig production in B⁻NOD mice. The I-A_β gene of the class II molecules in the parental B⁻NOD mouse was determined to be I-A_β^7, which is a unique class II gene of original NOD mice (20), according to the PCR findings (Fig. 1c).

Development of diabetes in B⁺NOD and in B⁻NOD mice

The development of diabetes in wild-type (+/+), μMT/+ B⁺NOD and μMT/μMT B⁻NOD mice was followed for 40 weeks. Every 3 weeks blood samples were monitored for blood glucose levels and diabetes was diagnosed when the blood glucose levels were >200 mg/dl. As shown in Fig. 2, diabetes developed in wild-type (+/+) and μMT/+ B⁺NOD mice beginning at 15 weeks of age and 87.8% (43 of 49) of B⁺NOD mice developed diabetes up to 40 weeks of age. In contrast, none (of 13) of the B⁻NOD mice developed diabetes by 40 weeks of age. Thus, the development of diabetes was prevented in B⁻NOD mice ($P < 0.0001$ as assessed by the Fisher's exact test), indicating that B cells are critically important for the development of diabetes in NOD mice.

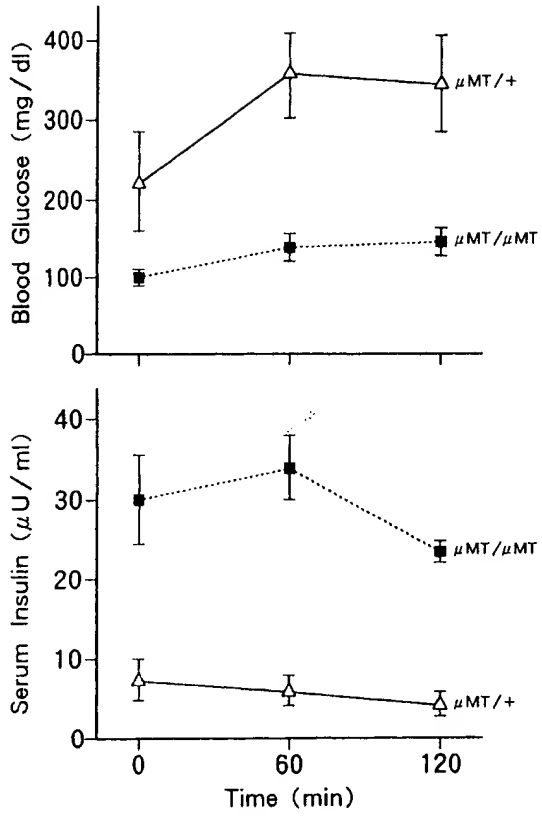

Fig. 3. Glucose and insulin reactivity in the B⁻NOD mice (μMT/μMT) and control B⁺NOD (μMT/+) mice after glucose loading. All data are the means ± SEM, $n = 3$ per group. Open triangles, μMT/+ B⁺NOD mice; closed squares, μMT/μMT B⁻NOD mice.

Glucose response and insulin reactivity after glucose loading in B⁺NOD and in B⁻NOD mice

B⁺NOD mice as well as the B⁻NOD mice were i.p. challenged with 1 g/kg body wt of glucose at 40 weeks of age. The blood glucose levels of the B⁺NOD mice showed a diabetic pattern >200 mg/dl at 120 min, while the B⁻NOD mice maintained normal glucose levels (Fig. 3). Although the insulin reactivity of B⁺NOD mice was significantly impaired, the B⁻NOD mice showed a good insulin response (Fig. 3), thus suggesting pancreatic β cell function to be well preserved in B⁻NOD mice.

Development of mild insulitis and suppression of the progression of insulitis in B⁻NOD mice

Although the development of mild insulitis was observed in B⁻NOD mice at 12 weeks of age, the extent of insulitis was lower in B⁻NOD mice than in B⁺NOD mice (Table 1). B⁻NOD mice showed mild insulitis at 30 weeks of age, at which time mild peri-insular mononuclear cell infiltration often associated with mild lymphocytic infiltration into the islets was seen (Fig. 4, left). In contrast, B⁺NOD mice developed moderate to severe insulitis at the same age, in which moderate mono-

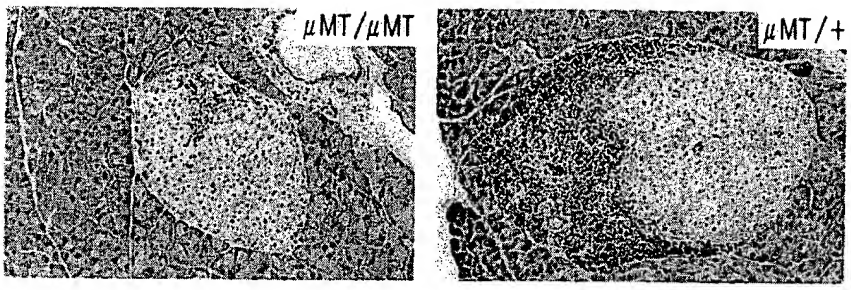

Fig. 4. Representative histopathology of insulitis in a B⁻NOD (μMT/μMT) mouse and a B⁺NOD (μMT/+) mouse at 30 weeks of age. Left: mild insulitis (insulitis score: 1) of a B⁻NOD (μMT/μMT) mouse: hematoxylin & eosin, ×200. Right: moderate insulitis (insulitis score: 2) of a B⁺NOD (μMT/+) mouse: hematoxylin & eosin, ×200.

Table 1. Incidence and grading of insulitis in B⁻NOD mice and B⁺NOD control littermates

Mice	Age (weeks)	No. of islets analyzed	Total no. of islets graded (percent of total): insulitis score			
			0	1	2	3
B⁺NOD (μMT/+)	12	300	99 (33.0)	77 (25.7)	67 (22.3)	57 (19.0)
B⁻NOD (μMT/μMT)	12	300	230 (76.7)	42 (14.0)	24 (8.0)	4 (1.3)
B⁺NOD (μMT/+)	30	300	80 (26.7)	68 (22.7)	79 (26.3)	73 (24.3)
B⁻NOD (μMT/μMT)	30	300	246 (82.0)	44 (14.7)	9 (3.0)	1 (0.3)

nuclear cell infiltration into the islets producing a lymphfollicle-like appearance (Fig. 4, right). Consistently, the insulitis scores of the B⁻NOD mice were significantly lower ($P < 0.0001$ as assessed by the Mann–Whitney test) than those of the B⁺NOD mice at either 12 or 30 weeks of age (Table 1), thereby indicating that the progression of insulitis was significantly suppressed in B⁻NOD mice.

Discussion

The present study demonstrates that diabetes did not develop during the 40 weeks observation period in B cell-deficient NOD mice, while as high as 90% of control NOD mice with B cells developed diabetes. In addition, although the B cell-deficient NOD mice did develop insulitis, the extent of insulitis was significantly suppressed, thereby indicating that B cells are crucial for the progression of insulitis as well as the development of diabetes in NOD mice.

Recently, Serreze *et al.* that reported neither insulitis nor diabetes developed in B cell-deficient (μMT/μMT) NOD mice, drawing a conclusion that B cells were required for the initiation of T cell-mediated autoimmunity in NOD mice (21). They interpreted the data that B cells might play an important role, at least as antigen-presenting cells to T cells in the initiation of insulitis (21). In this study, we demonstrated that mild insulitis was present in the B⁻NOD mice as early as 12 weeks of age, although the extent of insulitis was limited. Accordingly, it is suggested that initial activation of T cells against islets might occur, but further expansion of T cells might be impaired in B⁻NOD mice. Since antigen-presenting cells such as macrophages and dendritic cells are intact in B cell-deficient mice, the initial activation of T cells would be operative in B cell-deficient mice. Consistently, previous studies have reported that T cells were normally primed with peptide or parasite antigens in B cell-deficient mice and that peripheral tolerance was also successfully achieved (22–24). The reason for the discrepancy regarding the development of insulitis in our study and the previous study is not clear. This may be because they evaluated the insulitis only at 20 weeks of age, when insulitis may have not become evident or disappeared in the B⁻NOD mice they used. Alternatively, this may be due to differences in some unidentified genetic background between mice they used and ours.

Although the development of mild insulitis in B⁻NOD mice was observed in our study, progression of insulitis was significantly suppressed. Since it was reported that T cells predominantly infiltrated into the insulitic lesion of NOD mice (6,7), the expansion of T cells might be suppressed in B⁻NOD mice. We found the predominant accumulation of CD4⁺ T cells in the insulitic lesion of islets in B⁺NOD mice (K. Anzai *et al.*, manuscript submitted), while the proliferation of the CD4⁺ T cells in the insulitic lesion was prominently suppressed in B⁻NOD mice. Recently, several reports have indicated the critical role of B cells in the expansion of CD4⁺ T cells (23–25). In a parallel study, we created B cell-deficient B6 *lpr* mice, and found both lymphadenopathy and lymphoproliferation to be significantly suppressed in such animals, concomitant with the suppression of proliferation of CD4⁺ T cells (I. Akashi *et al.*, manuscript submitted). Based on these data, it is reasonable to postulate that the initial activation of T cells might be preserved in B⁻NOD mice, while the expansion of autoreactive CD4⁺ T cells might be impaired, preventing a further progression of insulitis.

It was reported that anti-B7-2 (a CD28 ligand)-mediated co-stimulatory signal through CD28 expressed on T cells plays an important role in the proliferation of autoreactive T

cells in NOD mice (26). Indeed, recent advances suggested that B7-1- and B7-2-mediated co-stimulatory signals play a role in the proliferation and differentiation of T cells (27). In addition, it was also recently reported that CD40 engagement by the CD40 ligand (CD40L) expressed on antigen-activated T cells is critical for the up-regulation of B7 molecules on antigen-presenting B cells for both T cell proliferation and differentiation (28). These observations taken together suggest the presence of a T cell–B cell augmentation circuit mediated by T cell–B cell interaction, which is operative by CD40L–CD40 ligation, followed by the up-regulation of B7 molecules on B cells, with the subsequent stimulation of T cells via CD28 expressed on T cells. Accordingly, the prevention of progression of severe insulitis in B$^-$NOD mice in our study might be due to the lack of B cell-mediated co-stimulatory signals which might be essential for the late activation and/or proliferation of autoreactive T cells against pancreatic β cells.

The question as to whether or not antigen-specific clonal T cell expansion was regulated by B cells should be clarified in further studies, since clonal T cells specific for islet cell antigen could successfully transfer diabetes in NOD mice (12–14). Another possible contribution of B cells in NOD diabetes is that B cells may play an important role as antigen-presenting cells, especially in the shift of the T cell repertoires which are responsible for the development of diabetes from the stage of mild insulitis to severe insulitis (29,30). It is also possible that autoantibodies produced by B cells against certain islet β cell-associated antigens may contribute to the progression of diabetes (16).

Although further detailed analysis is required to clarify the precise role of B cells in the pathogenesis of NOD mice, the present study demonstrates that B cells are essential for the progression of autoimmunity in NOD mice, thus providing new insight into the development of autoimmune diseases with respect to the role of B cells in addition to autoantibody production. A better understanding of the role of B cells in autoimmunity will therefore help to promote the development of new treatment strategies for autoimmune diseases using B cell-directed therapy.

Acknowledgements

The authors thank Dr Jiyang Wang for his helpful advice in conducting this research. This work is supported by a grant no. 8671170 from the Ministry of Education, Science and Culture of Japan.

Abbreviations

CD40L	CD40 ligand
NOD	non-obese diabetic mice

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CD40 Ligand-CD40 Interactions Are Necessary for the Initiation of Insulitis and Diabetes in Nonobese Diabetic Mice[1]

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The nonobese diabetic (NOD) mouse spontaneously develops T cell-dependent autoimmune diabetes. Here, we investigate the role of CD40 ligand (CD40L)-CD40 costimulation in the initiation and progression of this disease. Anti-CD40L mAb treatment of 3- to 4-wk-old NOD females (the age at which insulitis typically begins) completely prevented the insulitis and diabetes. In contrast, treatment of such mice with anti-CD40L at >9 wk of age did not inhibit the disease process. These results suggest that a costimulatory signal by CD40L is required early but not in the effector phase of disease development. Anti-CD40L treatment affected the priming of islet Ag-specific T cell responses in vivo. Cytokine analysis revealed a dramatic decrease in IFN-γ and IL-2 release without a concomitant increase in IL-4 production by T cells from anti-CD40L-treated mice. Thus, anti-CD40L impaired the islet Ag-specific Th1 cell response in vivo, and the prevention of diabetes by anti-CD40L was not associated with switching of the response from a Th1 to a Th2 profile. Cotransfer of splenocytes from anti-CD40L-treated mice with splenocytes from diabetic NOD mice into NOD/*scid* mice did not inhibit the transfer of disease, indicating that anti-CD40L does not prevent the disease by inducing regulatory cells. Since anti-CD40L clearly prevented the insulitis by inhibiting the development and further accumulation of pathogenic Th1 cells to islets of Langerhans, we conclude that CD40L-CD40 costimulation is required for early events in the development of spontaneous autoimmune diabetes. *The Journal of Immunology,* **1997, 159: 4620–4627.**

T cell activation by APCs involves costimulation through multiple receptor-counter receptor pairs: CTLA4/CD28-B7, LFA-1/ICAM-1, and CD40-CD40 ligand (CD40L).[3] CD40L is expressed on activated $CD4^+$ T cells, whereas CD40, the receptor for CD40L, is expressed on such varied APCs as B cells, dendritic cells, and macrophages (1). CD40-CD40L interactions can influence many aspects of T cell-mediated inflammatory responses, as reviewed in detail elsewhere (2). Triggering via CD40 increases the Ag-presenting function of B cells (3) and upregulates expression of the costimulatory molecules B7-1 and B7-2 (4, 5) as well as CD44H (6). CD40 signaling also activates macrophages to produce inflammatory cytokines (7) and reactive nitrogen intermediates (7, 8). In turn, CD40L is important for in vivo activation and clonal expansion of Ag-specific T cells (9).

Previously, CD40-CD40L interactions were found essential for the development of several autoimmune diseases such as collagen-induced arthritis (10), autoimmune oophoritis (11), and experimental allergic encephalomyelitis (12, 13). The role of CD40L-CD40 was also shown in Ab-dependent spontaneous systemic autoimmunity of MRL/*lpr* mice (14–16). However, the possibility that these molecules participate in T cell-dependent spontaneous autoimmune disease remains untested.

An example of this disease is the insulin-dependent diabetes mellitus (IDDM) of NOD mice, which develop insulitis by 3 to 5 wk of age (17) followed by diabetes at 3 to 7 mo of age, when destruction of β cell mass causes glucose levels to rise (18). Previously, after demonstrating that the blockade of CD28/B7 costimulation inhibited the genesis of diabetes but not insulitis, Lenschow et al. (19) suggested that other costimulation pathways may promote both lesions. Therefore, we tested here the activity of CD40L-CD40 in the insulitis and diabetes of NOD mice, the animal model of human IDDM.

Materials and Methods

Mice

NOD/*shi* mice were procured from the rodent breeding colony at The Scripps Research Institute, La Jolla, CA. These mice were maintained under pathogen-free conditions at this facility.

Antibodies

The B cell hybridoma-secreting hamster anti-mouse CD40L mAb (HB11048: MR1) was obtained from American Type Culture Collection, Rockville, MD. Its source was ascites fluid generated in pristane-primed, irradiated (600 rads), and anti-lymphocyte serum-treated (C57BL/6 × CBA/j) F_1 mice (The Jackson Laboratory, Bar Harbor, ME). The mAb concentration in the ascites fluid was determined by quantitative ELISA. Hamster IgG (Hlg) used as a control was purchased from Pierce (Rockford, IL). All Ab samples were tested for *Mycoplasma* organisms by ELISA

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Received for publication April 3, 1997. Accepted for publication August 4, 1997.

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[1] B.B. is supported successively by postdoctoral fellowships from the Juvenile Diabetes Foundation International (JDFI) and the Myasthenia Gravis Foundation of America, Inc. This work was supported by a grant from the Diabetes Interdisciplinary Research Program of JDFI (to N.S.), National Institutes of Health Grant DK33880 (to H.O.M.), and a research award from JDFI (to R.T.). This is manuscript no. 10522-NP from The Scripps Research Institute.

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[3] Abbreviations used in this paper: CD40L, CD40 ligand; GAD, glutamic acid decarboxylase; Hlg, hamster immunoglobulin; HSP, heat-shock protein; NOD, nonobese diabetic; H&E, hematoxylin and eosin; BG, blood glucose; IDDM, insulin-dependent diabetes mellitus.

0022-1767/97/$02.00

Table I. *Early but not late administration of anti-CD40L mAb prevents the development of spontaneous autoimmune diabetes in NOD/shi mice*

In vivo[a]	No. of Mice/ Group	Age at Start (weeks)	Age at Termination (weeks)	No. of Mice with Diabetes/ Total No. of Mice
Group A				
Anti-CD40L	6	3	31	0/6 (0%)
Hlg	5	3	31	4/5 (80%) ($p = 0.0128$)
PBS	8	3	31	6/8 (75%) ($p = 0.0077$)
Group B				
Anti-CD40L	6	3	24	0/6 (0%)
Hlg	5	3	24	4/5 (80%) ($p = 0.0128$)
Group C				
Anti-CD40L	6	9–10	29	4/6 (67%)
Hlg	5	9–10	29	5/5 (100%) ($p = 0.231$)

[a] The female NOD/*shi* mice of indicated ages were treated with anti-CD40L, Hlg, or PBS i.p. as described (see *Materials and Methods*). The BG levels were measured once in 2 wk when mice reached 15 wk of age. Mice were considered diabetic when the BG levels were > 300 mg/dl. *p* values were calculated using the log-rank (Mantel-Cox) test.

using a *Mycoplasma* detection kit (Boehringer Mannheim, Mannheim, Germany) and proved to be negative (data not shown).

Antibody treatment of mice

Treated subjects were NOD mice injected i.p., starting at 3 wk of age, on alternate days (0, 2, and 4) with 250 μg of anti-CD40L mAb or Hlg in 250 μl of PBS. The injections were repeated at 6, 9, and 12 wk of age as above. The Ab injections were then repeated at 21-day intervals since the half-life of the anti-CD40L in vivo is between 12 days and 21 days. In a preliminary experiment, we found that this treatment protocol was effective in preventing insulitis. Elsewhere, anti-CD40L was injected once in 2 days or 4 days throughout the experiment to block collagen-induced arthritis (10) and to investigate the role of CD40L in thymic selection of T cells (20).

Measurement of blood glucose (BG) levels

In blood samples obtained from the retro-orbital sinuses of anesthetized mice, beginning when they were 15 wk old, BG levels were determined every 2 wk using an AccuChek II monitor (Boehringer Mannheim Diagnostics, Indianapolis, IN). Mice were considered diabetic when the BG levels were >300 mg/dl.

Histologic analysis

For evaluation of peri-insulitis and insulitis, pancreata from anti-CD40L-treated and control mice were fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer-thick multiple sections of the tissue were cut and then stained with hematoxylin and eosin (H&E). Acetone-fixed cryostat sections were stained with anti-Mac-1 (Boehringer Mannheim), anti-B220, anti-CD4, and anti-CD8 (PharMingen, San Diego, CA) mAb. After incubation with biotinylated secondary Ab (Vector Laboratories, Inc., Burlingame, CA), sections were exposed to avidin-biotin-peroxidase complex (Vector Laboratories). After the color reaction with diaminobenzidine, the sections were counterstained with hematoxylin as described previously (21).

T cell proliferation in response to islet Ags

Single-cell splenocytes obtained from anti-CD40L or control group mice were cultured (4×10^5/well) with and without Ags in HL-1 serum-free medium (BioWhittaker, Walkersville, MD) in 96-well flat-bottom microculture plates for 4 days. As a source of Ags, irradiated islet cells (2×10^4/well), GAD65 (20 μg/ml), and HSP65 (5 μg/ml) were used. The cDNAs for the production of recombinant GAD65 and HSP65 were provided by H. O. McDevitt (Stanford University). The cultures were pulsed during the last 18 h of the assay and were later harvested as described (22). The values were expressed as a stimulation index ± SD; values >3 are considered significant.

Cytokine assays

Splenocytes taken from 12-wk-old NOD mice that had been treated (starting when the mice were 3–4 wk of age) with anti-CD40L or control Ab were prepared in single-cell suspensions as described above and placed in sterile PBS. These splenocytes were then treated with a red cell lysis buffer and resuspended in mouse tonicity PBS. T cells were isolated by nonadherence to nylon wool. Next, these purified T cells (2×10^6) were stimulated in vitro with plate-bound anti-αβ TCR mAb (5 μg/ml) (clone H57-597, PharMingen) in serum-free HL-1 medium. The culture supernatants were collected at 24 h (for IL-2 measurements) and 48 h (for IL-10 and IFN-γ measurements). The samples were frozen at −20°C. The cytokine content of the supernatants was determined using an ELISA kit as recommended by the manufacturer (PharMingen).

Adoptive transfer of diabetes

To establish the effect of anti-CD40L mAb on the induction of regulatory lymphocytes, cotransfer experiments were performed. Female NOD/*scid* mice age 11 to 12 wk were injected i.v. with 2×10^7 splenocytes in 0.2 ml of sterile PBS containing a 1:1 ratio of splenocytes from recently diabetic NOD female mice and 12-wk-old nondiabetic anti-CD40L- or control Ab (Hlg)-treated mice. In some experiments, NOD mice were treated with anti-CD40L or control Ab beginning at 3 wk of age. At 12 wk of age, these mice were irradiated with 700 rads from a cesium source and injected with 2×10^7 splenocytes in 0.2 ml of sterile PBS from recently diabetic NOD females. Their BG levels were measured at weekly intervals.

Statistical analysis

Statistical analyses were performed using the log-rank test or Student's *t* test.

Results

Early treatment of NOD mice with anti-CD40L mAb prevents their characteristic autoimmune diabetes

To determine the effect of CD40-CD40L interactions on the development of spontaneous autoimmune diabetes, NOD mice were treated, starting at 3 wk of age, with hamster anti-CD40L ($n = 6$), control Hlg ($n = 6$), or PBS ($n = 8$) alone. The mice were killed at 24 wk and 31 wk of age, the time points when the cumulative incidence of diabetes reaches between 75% to 80% for NOD females in our specific pathogen-free NOD/*shi* colony. The results are summarized in Table I. As shown in group A, anti-CD40L completely blocked the development of autoimmune diabetes (0/6 = 0%), whereas neither Hlg nor PBS arrested the diabetes, denoted by BG levels >300 mg/dl in 80% (4/5) and 75% (6/8), respectively, of the latter two groups. In the Hlg-treated group, one mouse died from anesthesia at the time of BG estimation and was excluded. In a second experiment (group B) terminated at 24 wk, none (0% = 0/6) of the anti-CD40L treated NOD mice developed diabetes, whereas 80% (4/5) of control (Hlg-treated) mice developed diabetes, confirming the effectiveness of the anti-CD40L in prevention of diabetes. Therefore, CD40-CD40L interactions seemed to be critical in the development of spontaneous diabetes.

Early treatment of NOD mice with anti-CD40L mAb retards insulitis in NOD mice

Because insulitis usually precedes diabetes in NOD mice (23), we questioned whether anti-CD40L-treated mice would develop insulitis despite the absence of diabetes. After H&E staining and histologic examination, pancreata from anti-CD40L-treated mice (31 wk of age) revealed no insulitis (Fig. 1*A*), whereas pancreata from matched controls showed, as expected, either severe insulitis or complete destruction of islets (Fig. 1*B*). Normal islet architecture and intact islet mass in the pancreatic sections from anti-CD40L-treated mice were further confirmed by insulin staining (data not shown). Scores enumerating the severity of inflammatory infiltrates in both groups appear in Table II. As shown (group A), significantly greater percentages of islets were intact among pancreata from anti-CD40L-treated mice (93%) than in those from the

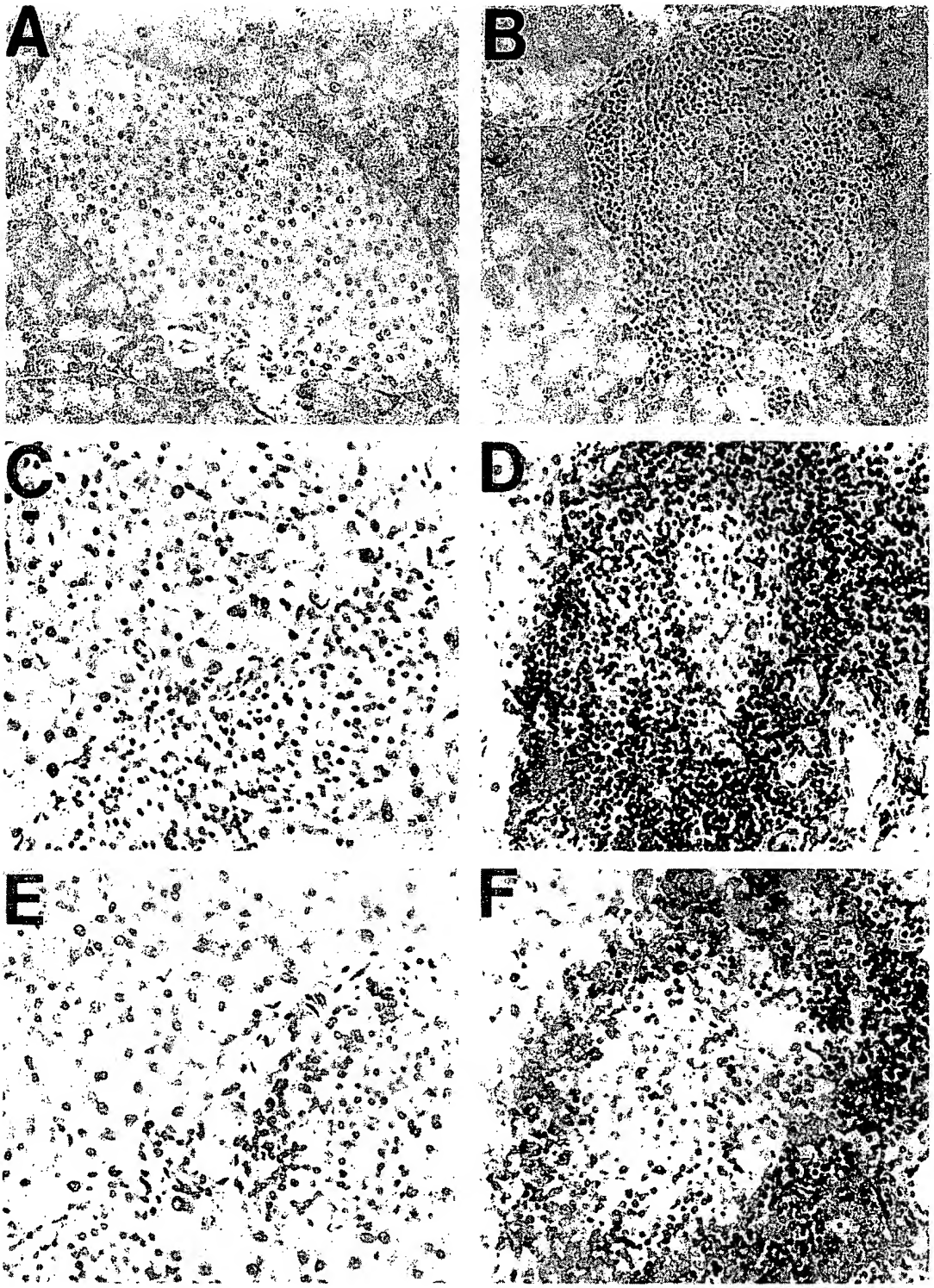

FIGURE 1. Histologic analysis of representative H&E-stained, paraffin-embedded sections from pancreata of 31-wk-old (*A*) anti-CD40L-treated NOD mice, which show no insulitis, and (*B*) HIg-treated control mice with severe insulitis (×200). Immunohistochemical staining of pancreata from 8-wk-old anti-CD40L- treated NOD mice did not contain (*C*) $CD4^+$ or (*E*) $CD8^+$ T lymphocytes. In contrast, pancreata from age-matched control contains a heavy infiltrate with (*D*) $CD4^+$ and (*F*) $CD8^+$ T lymphocytes (×200). I. denotes islets of Langerhans in the pancreas.

control mice (2–12%). In a second experiment (Table II, group B), H&E staining of pancreata from anti-CD40L-treated NOD mice of 24 wk of age did not reveal any lymphocytic infiltration (peri-insulitis or insulitis) (0% of the 482 islets scored), whereas 78% of the islets (133 islets scored) of control mice showed lymphocytic infiltration. Furthermore, immunoperoxidase staining of pancreatic sections from anti-CD40L-treated NOD mice at 8 wk of age revealed no infiltration by either $CD4^+$ or $CD8^+$ T cells (Fig. 1, *C* and *D*), whereas pancreata from control HIg-treated mice showed heavy infiltrates (Fig. 1, *E* and *F*). In the latter group, the infiltrates

Table II. *Histologic analysis of pancreatic sections from anti-CD40L-treated and control NOD mice*

Group[a]	Mice (n)	Age at Start (weeks)	Histologic Analysis of Pancreatic Islets				
			Total	Normal	Peri-insulitis	Insulitis	Islet remnants
Group A							
Anti-CD40L	(n = 6)	3	127	118 (93%)[b]*	9 (7%)	0 (0%)*	0 (0%)*
Hlg	(n = 5)	3	115	14 (12%)	24 (21%)	56 (49%)	21 (18%)
PBS	(n = 8)	3	121	2 (2%)	12 (10%)	68 (56%)	39 (32%)
Group B							
Anti-CD40L	(n = 6)	3	482	482 (100%)[b]*	0 (0%)	0 (0%)*	0 (0%)*
Hlg	(n = 5)	3	133	28 (21%)	64 (48%)	41 (30%)	ND
Group C							
Anti-CD40L	(n = 6)	9–10	154	1 (0%)**	24 (15%)	115 (75%)**	14 (10%)**
Hlg	(n = 5)	9–10	247	13 (5%)	34 (14%)	185 (75%)	15 (6%)

[a] Anti-CD40L or control Ab were administered to NOD mice as described in *Materials and Methods*. The experiment was terminated at 31 wk (group A), at 24 wk (group B), and 29 wk (group C) of age. The paraffin-embedded sections were stained by H&E. Approximately 10 to 20 islets were scored for each pancreas. Note that mice (anti-CD40L-treated) in Group A had islets with no insulitis or very few islets with peri-insulitis and a higher proportion of normal islets.

[b] Percentage of islets was calculated by the following formula: (number of islets normal, with peri-insulitis or insulitis or completely damaged/total number of islets). ND denotes not done. * Denotes values significantly different from those of controls ($p < 0.05$); ** denotes values not significantly different from those of controls ($p > 0.05$).

Table III. *The effect of anti-CD40L on spontaneous autoreactive T cell response in NOD/shi mice*

Mouse No.	In vivo	In vitro Lymphocyte Proliferative Response (Stimulation Index ± SD) to				
		Bcgr.cpm	Islet Ag	GAD65	HSP65	Control lysate
1	Anti-CD40L	5813	7.7 ± 0.0[b]	5.3 ± 0.7[b]	7.0 ± 0.4[b]	1.9 ± 0.1
2	Anti-CD40L	3259	8.2 ± 1.4[b]	5.1 ± 0.9[b]	7.1 ± 0.8[b]	1.6 ± 0.2
3	Hlg	2514	14.9 ± 1.0	9.7 ± 0.5	11.3 ± 1.8	2.1 ± 0.1
4	Hlg	1621	14.0 ± 1.6	11.3 ± 1.2	13.0 ± 0.9	2.0 ± 0.1

[a] Splenocytes from individual NOD/*shi* mice of experimental and control groups were isolated and cultured at 4×10^5 well in 200 μl of serum-free HL-1 medium (BioWhittaker) in 96-well flat-bottom microculture plates in the presence of the indicated Ag for 4 days. The cultures were pulsed during the last 18 h of the assay and were later harvested as described (22). Stimulation index values > 3 are considered significant.

[b] The differences in proliferative responses against various islet Ags between two groups (anti-CD40L vs Hlg) are statistically significant. p values (anti-CD40L vs Hlg groups) for: Islet Ag, 0.006; GAD65, 0.021; and HSP65, 0.026. p values were calculated using Student's t test (unpaired).

also consisted of B cells and macrophages (data not shown). Thus, anti-CD40L treatment effectively prevented the development of insulitis.

Late treatment with anti-CD40L mAb does not affect the development of spontaneous autoimmune diabetes and insulitis in NOD mice

Although the first evidence of insulitis (infiltration of lymphocytes) can be detected in mice as young as 3 to 4 wk of age (24), full-blown diabetes does not develop until much later. Therefore, at least two distinct events seem to participate in the eventual clinical disease. To examine the effect of anti-CD40L given well after the induction of insulitis, but before hyperglycemia or any other sign of clinical disease appeared, we delayed treatment of NOD mice until they were 9 to 10 wk old. Injections of anti-CD40L or Hlg three times in the first wk and again 21 days later failed to block the development of autoimmune diabetes, respectively, in 4/6 (67%) and 5/5 (100%) of the mice (Table I). Despite the ability of anti-CD40L to circumvent disease completely when administered early, it had far less effect later in the disease process.

H&E-stained sections of pancreata from anti-CD40L-treated mice (29 wk of age) revealed extensive insulitis similar to those pancreata from matched control group mice. Scores enumerating the severity of inflammatory infiltrates in both groups appear in Table II (group C). As shown, no significant differences were observed in percentages of islets with insulitis among pancreata from anti-CD40L-treated mice (75%) and the control mice (75%).

Anti-CD40L mAb impairs the priming of islet Ag-specific T cell responses

Female NOD mice exhibit spontaneous T cell reactivity to islet Ags such as GAD 65 and HSP65 (25–27). Additionally, CD40L-CD40 interactions are necessary for in vivo priming of T cells and for the initiation of specific T cell immune responses (9). Therefore, to test whether the inhibition of insulitis and diabetes by anti-CD40L in NOD mice originates from impaired T cell priming to the islet Ags, we performed lymphocyte proliferation assays. NOD mice at 3 wk of age were treated with anti-CD40L (n = 2) or Hlg (n = 2). Ab treatment was repeated 21 days later. The mice were killed at 8 wk of age, and their splenocytes were tested for proliferation in response to irradiated islet cells, GAD65, and HSP65. As a result (Table III), T cells from anti-CD40L-treated and control mice proliferated vigorously against indicated islet Ags. Although vigorous proliferation of lymphocytes from anti-CD40L-treated mice to islet Ags was noticed, there is a reduction (<2-fold) in the proliferation of these cells as compared with those of control mice. These results indicate that anti-CD40L treatment partially impairs the T cell priming; however, it did not inactivate islet-reactive T cells.

Anti-CD40L treatment impairs the priming of islet Ag-reactive Th1 T lymphocytes

To learn whether the protection of NOD mice from insulitis and diabetes by anti-CD40L results from a switch from Th1 to Th2 responses, as previously reported (28), we analyzed the cytokine profile of NOD T lymphocytes.

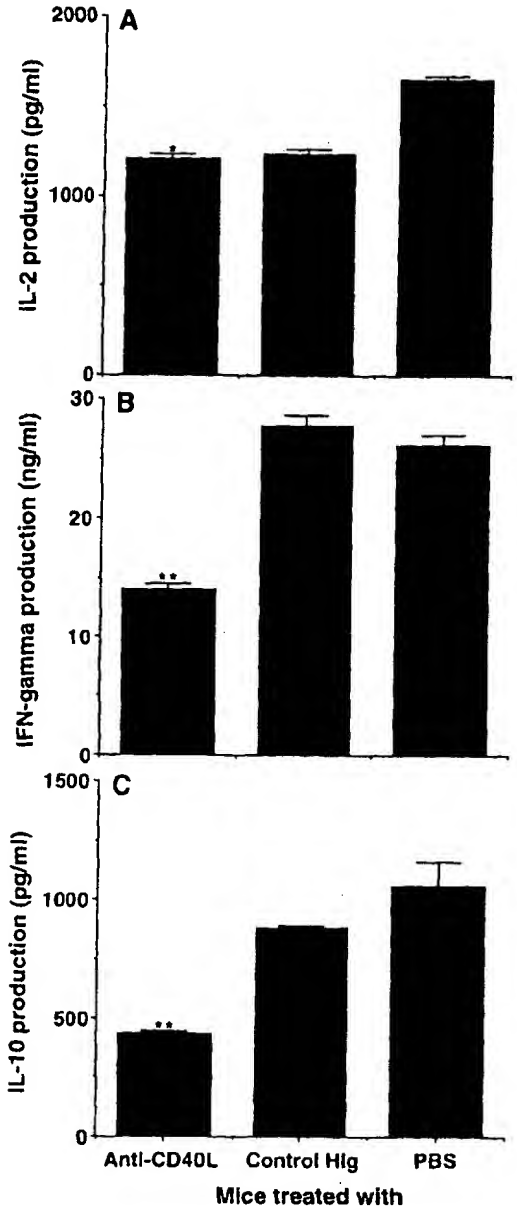

FIGURE 2. Cytokine profile of T cells from anti-CD40L and control mice. The mice (n = 2) were killed at 12 wk of age after treatment, starting at 3 wk of age, with anti-CD40L, control Hlg, or PBS. The Ab treatments were repeated at 21-day intervals. The cytokine levels ((*A*) IL-2, (*B*) IFN-γ, and (*C*) IL-10) were determined as outlined in *Materials and Methods*. p values were calculated between anti-CD40L vs Hlg; anti-CD40L vs PBS groupAs. **, Indicates $p < 0.05$; *, $p > 0.05$.

Nylon wool-purified T cells from anti-CD40L-treated mice or control mice at 12 wk of age were boosted with anti-TCR mAb in vitro. As shown in Figure 2, analysis of cytokine levels by ELISA revealed that T cells from anti-CD40L-treated mice produced significant quantities of Th1 (IL-2 and IFN-γ) and Th2 (IL-10) cytokines. Levels of IL-2 were similar in the T cell cultures of anti-CD40L and controls, although the levels of IFN-γ and IL-10 were significantly (approximately twofold) lower for the anti-CD40L-treated group compared with the control group. A reduction in the levels of IFN-γ and IL-10 by lymphocytes may be a consequence of partial impairment in the proliferative responses of these cells in anti-CD40L-treated mice. Since IFN-γ and IL-10 cytokines were detected in the culture supernatants of anti-CD40L-treated mice, these findings suggest that no switch in the T helper subset profile was associated with the prevention of insulitis and diabetes in the anti-CD40L-treated mice.

In addition, to assess the cytokine production more precisely by islet Ag-specific T cells, we stimulated the splenocytes from anti-CD40L mice and control mice with islet Ags (irradiated whole islet cells). Splenocytes from anti-CD40L-treated mice produced very little amounts of IFN-γ (0.221 ng/ml) and IL-2 (23.7 pg/ml). In contrast, the splenocytes control group of mice readily produced IFN-γ (25.02 ng/ml) and IL-2 (53.2 pg/ml). However, no difference in IL-4 (CD40L vs control: 31.6 pg/ml and 29.9 pg/ml) and IL-10 (CD40L vs control: 52.3 pg/ml vs 45.5 pg/ml) production by islet Ag-specific T cells was observed. These findings suggest that the prevention of insulitis and diabetes in the anti-CD40L-treated mice is not associated with switching of the islet Ag-specific Th1 subset to Th2 subset. Rather, the current findings indicate that anti-CD40L treatment of NOD mice inhibits development of islet Ag-specific Th1 cells in vivo (not shown).

Anti-CD40L treatment of NOD mice does not evoke regulatory activity

To test directly whether the inhibitory effects of anti-CD40L on autoimmune insulitis and diabetes emanates from the induction of disease suppressing immunoregulatory cells in vivo, we performed cotransfer experiments. Splenocytes (1×10^7) from anti-CD40L- or control Hlg-treated NOD mice were mixed with splenocytes (1×10^7) from NOD females that had become diabetic within the previous two wk, then transferred into 11- to 12-wk-old female NOD/*scid* recipients (Table IV). Regardless of whether the splenocytes originated from anti-CD40L mAb (8/8)-, or control Hlg (7/7)-treated mice, all NOD/*scid* recipients developed overt diabetes within 6 wk post-transfer. The incidence of disease did not differ significantly when cotransferred splenocytes came from anti-CD40L-treated mice or Hlg-treated mice. Therefore, the protection mediated by anti-CD40L mAb did not rely on the induction of immunoregulatory cells in vivo.

Anti-CD40L does not influence damage to islets by effector cells

To determine whether anti-CD40L treatment of NOD mice protects them from autoimmune diabetes caused by effector cells, we performed adoptive transfer experiments. Recipients were 12-wk-old NOD females previously treated with anti-CD40L (n = 4) or control Hlg (n = 4), then irradiated and given adoptive transfers of splenic cells from diabetic mice. In all these recipients, diabetes arose rapidly (Table V), indicating that anti-CD40L treatment did not block the functions of effector cells already primed optimally in vivo.

Discussion

Upon examining the role of CD40L-CD40 costimulation in autoimmune diabetes, we found that the administration of anti-CD40L blocked the development of insulitis and diabetes, the prototypic autoimmune lesion of NOD mice. The development of islet-reactive Th1 cells is impaired without a concomitant switch to Th2 cells. However, T cell autoreactivity was easily demonstrated.

Previously, CD28/B7 and LFA/ICAM-1 were cited as the critical costimulation pathways in the immunopathogenesis of autoimmune diabetes. For example, ectopic expression of the costimulatory molecule B7-1 on islets of Langerhans accelerated autoimmune diabetes in NOD mice (29). Elsewhere, in a more

Table IV. *The effect of cotransfer of splenocytes from anti-CD40L-treated NOD with diabetic NOD splenocytes to NOD-scid recipients*

Source of Splenocytes[a]	Recipients	Incidence of Diabetes (Weeks Post-transfer) 1	2	3	4	5	6
Anti-CD40L-treated NOD + Diabetic NOD mice	NOD/*scid*	2/8	2/8	3/8	3/8	4/8	8/8
Hlg-treated NOD + Diabetic NOD mice	NOD/*scid*	1/7	1/7	1/7	2/7	2/7	7/7

[a] 1×10^7 splenocytes from recent diabetic NOD mice were mixed with 1×10^7 splenocytes from 12-wk-old anti-CD40L- or Hlg-treated NOD mice and were injected i.v. into 11 to 12-wk-old NOD/*scid* recipient mice. Diabetes was monitored by BG analysis at weekly intervals. Mice were considered diabetic when the BG levels were >300 mg/dl.

Table V. *Transfer of diabetes by adoptive transfer of diabetogenic splenocytes into irradiated recipients*

Source of Cells	NOD Recipients[a]	Incidence of Diabetes (Weeks Post-transfer) 1	2	3	4	5	6	7
Diabetic NOD	Anti-CD40L *treated*	0/4	0/4	1/4	4/4			
Diabetic NOD	Control Hlg treated	1/4	3/4	3/4	3/4	3/4	3/4	4/4

[a] 2×10^7 splenocytes from recent diabetic NOD mice were injected i.v. to 12-wk-old anti-CD40L- or control mAb-treated mice. Diabetes was monitored by BG analysis at weekly intervals. Mice were considered diabetic when the BG levels were >300 mg/dl.

complex system, the ectopic expression of the B7-1 on islets induced autoimmune diabetes in mice that are additionally transgenic for the T cell receptor and the corresponding target Ag. However, expression of the B7-1 transgene on islets alone is not sufficient to induce diabetes (30). Additionally, through up-regulation of the B7-1 molecule, the expression of IFN-γ transgene in islets activated autoreactive T cells (31, 32), but elsewhere, up-regulation of B7-2 and ICAM-1 molecules following the ectopic expression of IFN-α in islets was deemed responsible for activating autoreactive T cells (33). Still others, using conventional Ab for blocking studies, showed that CD28/B7 (19) and ICAM-1/LFA-1 (34) costimulation pathways influenced the development of insulitis and diabetes in NOD mice (see Table VI).

Our rationale for testing the CD40L-CD40 pathway was results from previous studies in which the blockade of CD40L-CD40 interactions interfered with T cell-dependent B cell responses (35, 36), T cell priming (9), and diseases of autoimmune etiology (10, 12). Accordingly, we showed here that NOD mice treated with anti-CD40L before the expected onset of insulitis never developed either insulitis or diabetes (Tables I and II). Interestingly, CTLA4Ig or anti-B7-2 treatment, which affects CD28/B7 costimulation, protected NOD mice from diabetes but not insulitis (19) (see Table VI). Therefore, anti-CD40L and anti-B7-2/CTLA4Ig may inhibit the development of diabetes by distinct mechanisms or with different timing. That the blockade of CD40L-CD40 interaction interferes early in the disease process receives support here from two pieces of evidence: 1) anti-CD40L treatment initiated after the onset of insulitis (9–10 wk of age) failed to prevent diabetes in NOD mice (Tables I and II); 2) splenocytes from diabetic mice readily transferred diabetes to anti-CD40L-treated NOD mice (Table V). Similarly, the blockade of LFA-1/ICAM-1 costimulation by mAb abrogated the development of insulitis and diabetes in NOD mice (34) (see Table VI).

The prevention of insulitis and diabetes cited here do not entirely stem from impairment in the priming of β cell-reactive T cells, since splenocytes from anti-CD40L-treated NOD mice proliferated to islet Ags in vitro albeit less well than those from controls (Table III). Thus, in the absence of CD40L-CD40 engagement, self-reactive T cells apparently do exist in the periphery but fail to become pathogenic. In support of this, adoptive transfer of the splenocytes from anti-CD40L treated mice into NOD/*scid* mice failed to transfer disease (0/7), whereas splenocytes from control mice readily transferred the disease (3/5) (data not shown). Interestingly, inhibition of insulitis and diabetes in NOD mice treated with mAb that affect the LFA-1/ICAM-1 pathway is not accompanied by inhibition of T cell responses to islet Ags (34) (see Table VI). Therefore, anti-CD40L and anti-ICAM-1 mAb may inhibit the development of diabetes by distinct mechanisms.

Since T cells responded to islet Ags in our experiments, albeit at a lesser intensity, an alternative route for the prevention of insulitis and diabetes by anti-CD40L might have been the induction of immune deviation: that is, inhibition of pathogenic Th1 cell development and promotion of protective Th2 cell production, as shown in a model of hapten-induced colitis (28). Exogenous administration of IL-4 or expression of an IL-4 transgene in β cells also protected NOD mice from insulitis and diabetes (21, 37). However, our direct demonstration the absence of Th1 cytokines (IL-2 and IFN-γ) and no concomitant elevation in IL-4 and IL-10 production in T cell cultures from anti-CD40L-treated mice indicates no role for immune deviation in the protection from pathologic (insulitis) and clinical (diabetes) disease of NOD mice.

Furthermore, we discounted the possibility that immunoregulatory cells may have inhibited the insulitis and diabetes of anti-CD40L-treated mice since cotransfer of splenocytes from anti-CD40L-treated mice and diabetic NOD mice failed to prevent the development of diabetes in recipients (Table IV). Moreover, we continue to detect islet Ag-specific T cell reactivity in vitro, albeit at a lesser intensity (Table III), which should have been completely inhibited if immunoregulatory cells were involved as shown elsewhere (38). Additionally, islet Ag-reactive Th2 cells or clones have the potential to home to the islets of Langerhans and cause peri-insulitis or insulitis (39–41). In the present study, anti-CD40L-treated mice are free of peri-insulitis and insulitis (Table II and see Table VI).

The site of initial priming of β cell-reactive T cells in spontaneous IDDM in NOD mice remains elusive. After using cytokine-transgenic, μ chain-knockout, and drug-induced animal models of IDDM, others have suggested that initial priming of β cell-reactive T cells could occur either within (13, 21, 42) or outside (43, 44) the pancreatic environment. The data reported here support the contention that the priming of β cell-reactive T cells occurs outside the immediate pancreatic environment (43, 44). Furthermore, we suggest that the CD40-CD40L costimulation is required for that initial event. In contrast, it appears that CD28/B7 (45) and LFA-1/ICAM-1 (34) costimulation pathways are not required for that initial priming of T cells in vivo.

Table VI. *Role of CD28/B7, ICAM-1/LFA-1, and CD40/CD40L costimulation pathways in IDDM of NOD mouse*

Parameter	Effect of Blockade of Various Costimulation Pathways Using mAb		
	CD28/B7.2 costimulation	ICAM-1/LFA-1 costimulation	CD40/CD40L costimulation
Peri-insulitis	No effect (19)	No effect (34)	Prevented (present study)
Insulitis	No effect (19)	Prevented (34)	Prevented (present study)
Priming of islet-reactive T cells	No effect (45)[a]	No effect (34)	Partially affected (present study)
Regulatory cells	ND	Not generated (34)	Not generated (present study)
Cytokine release by T cells	ND	ND	Decrease in IFN-γ and IL-2 (Th1 priming impaired) (present study)
Stage of requirement	After insulitis but before the onset of diabetes (19)	Before the onset of insulitis (34)	Before the onset of insulitis (present study)
Mechanism(s) of action	Unknown (19)	Homing or inactiviation of diabetogenic T cells (?) (34)	Generation and homing of diabetogenic Th1 cells (present study)

[a] The data were obtained with the use of NOD mice that are homozygous for CD28 mutation (45). ND denotes not done.

Why the islet-reactive T cells failed to accumulate in the islets in anti-CD40L-treated mice is the central question. We hypothesize that anti-CD40L may preclude the entry of β cell-reactive T cells into the pancreas by preventing the interaction of activated T cells with vascular endothelial cells. In our preliminary experiments, VCAM-1 expression on the endothelium was weaker in the pancreatic sections of anti-CD40L-treated mice than in those of the control mice (not shown). In that regard, physical contact between $CD40L^+$ T cells and $CD40^+$ endothelial cells up-regulates the expression of ICAM-1 and VCAM-1 on endothelial tissues and increases their vascular permeability (46) owing to the inflammatory Th1 cytokine(s) such as IFN-γ (47). Therefore, based on the results (a dramatic prevention of insulitis and a decrease in IFN-γ production by T cells of anti-CD40L-treated mice), we suggest that the development of insulitis and diabetes in these mice may depend on the generation of islet Ag-specific pathogenic Th1 cells and their homing potential in vivo, implying the requirement for CD40-CD40L interaction between T cells and APC for these two processes.

From the foregoing results, we suggest that CD40-CD40L costimulation is required for the generation and homing of islet-reactive pathogenic Th1 cells to the pancreas and their further access to islet Ags; without such stimulation, the downstream events leading to diabetes cannot ensue. On the basis of our current evidence that CD40-CD40L costimulation is critical for the initial events of insulitis and diabetes, our results may provide a new prophylactic strategy for inducing permanent protection from IDDM in humans with high-risk genetic backgrounds.

Acknowledgments

We thank Phyllis Minick and Cecile King for editorial corrections on this manuscript. We also thank Dr. Howard Fox (Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA) for performing ELISA on the Ab samples for *Mycoplasma* organisms.

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PNAS
Proceedings of the National Academy of Sciences of the United States of America www.pnas.org

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Proc. Natl. Acad. Sci. USA
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Immunology

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(transplantation/tolerance/islets of Langerhans/diabetes mellitus)

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Communicated by Roger H. Unger, The University of Texas Southwestern Medical Center, Dallas, TX, June 22, 1995

ABSTRACT **Combined treatment with allogeneic small lymphocytes or T-depleted small lymphocytes plus a blocking antibody to CD40 ligand (CD40L) permitted indefinite pancreatic islet allograft survival in 37 of 40 recipients that differed from islet donors at major and minor histocompatibility loci. The effect of the allogeneic small lymphocytes was donor antigen-specific. Neither treatment alone was as effective as combined treatment, although anti-CD40L by itself allowed indefinite islet allograft survival in 40% of recipients. Our interpretation is that small lymphocytes expressing donor antigens in the absence of appropriate costimulatory signals are tolerogenic for alloreactive host cells. Anti-CD40L antibody may prevent host T cells from inducing costimulatory signals in donor lymphocytes or islet grafts.**

A long-term goal in allotransplantation is to develop methods of inducing antigen-specific tolerance to replace generalized immunosuppression. Recent advances in understanding how antigen-presenting cells (APCs) regulate T-cell activation now provide new strategies for inducing transplantation tolerance.

To proliferate and differentiate into destructive effector cells, T cells depend on multiple signals from APCs. APCs use nonclonal recognition systems to detect tissue damage or infectious organisms (1). These signals are termed costimulation (2). When an antigen receptor is ligated, responding T cells continuously monitor for these signals in the environment. Costimulatory signals include the interaction between CD28 on responding T cells and B7 family members on APCs. This interaction enhances autocrine interleukin 2 synthesis and secretion by the responding T cell (3). Costimulatory signals can also regulate effector functions elicited by antigen (4).

Only dendritic cells constitutively express high levels of costimulatory molecules (5, 6), and their expression can be further up-regulated (6, 7). On macrophages, B lymphocytes, endothelial cells, and keratinocytes, costimulatory molecules can be induced by infection, adjuvant, inflammatory cytokines, or activated T cells (3, 8–11).

T cells become anergic or die when they recognize antigen in the absence of costimulation *in vitro* (2, 12). T cells escaping clonal deletion in the thymus disappear or become anergic when they recognize self-antigens on healthy APCs *in vivo* (13, 14). This is thought to be a major mechanism of self-tolerance.

These observations suggest that tissue allografts should be tolerogenic if care is taken to avoid transplanting cells with constitutive costimulatory activity. The concept of costimulation was first proposed in the context of "passenger leukocytes" and allograft rejection (15), and its importance has been underscored by demonstrations that blockade of B7–CD28 interaction allows survival of cardiac allografts (16) and xenogeneic human islets (17) in mice. Blockade was achieved using CTLA4–Ig fusion protein, CTLA4 being a CD28-like receptor found on activated T lymphocytes (18).

Activated $CD4^+$ T cells express a membrane-bound protein, CD40 ligand (CD40L, gp39), which engages CD40 on resting B cells and accounts for most cell contact-dependent T-cell help for small B cells (19–21). CD40L induces expression of B7 and other costimulatory activities of B cells; it also enhances expression of costimulatory molecules on other APCs (9, 22).

Here we report the survival of allogeneic islet grafts in chemically diabetic mice pretreated with allogeneic small lymphocytes, an antibody to the CD40L to prevent T-cell–B-cell interactions, or both treatments in combination. When anti-CD40L was combined with transfusion of small lymphocytes of donor type, long-term islet graft survival was obtained in nearly all recipients.

MATERIALS AND METHODS

Mice. C57BL/6 ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$), (BALB/c × C57BL/6)F_1, and (C57BL/6 × C3H)F_1 mice were obtained from the National Cancer Institute, Frederick, MD. C3H mice are $H\text{-}2^k$. Animals were housed in microisolators and provided with autoclaved food and water *ad libitum*.

Preparation of Lymphocytes and Assays for APC Function. Spleen cells from 8-wk-old female mice were depleted of erythrocytes and fractionated by centrifugal elutriation (23, 24). A small cell fraction (fraction 19) was collected at 19 ml/min at 3200 rpm in a JE-6B rotor or at 77 ml/min at 3200 rpm with a JE-5.0 rotor (Beckman). A second fraction (fraction 20) was collected at 20 ml/min. The fractions were composed of small lymphocytes with a symmetric size distribution (mean cell volume, 115–120 μm^3; Coulter Counter and Multisizer; Coulter). In some experiments, small lymphocyte fractions were depleted of T cells by treatment with anti-T-cell monoclonal antibodies and complement (25). Before T-cell depletion, fraction 19 was reduced in T cells (39% $CD3^+$ in unfractionated spleen; 14% $CD3^+$ in fraction 19; mean of three experiments). The T-depleted fraction 19 cells were 89% $B220^+$, 1.1% $CD3^+$, and 10% null, presumably natural killer, cells. To monitor radioresistant APC function, fractionated cells were exposed to 3000 rads (1 rad = 0.01 Gy) from a ^{137}Cs source and tested for ability to induce proliferation of the $H\text{-}2^b$ alloreactive Th2 line, D10.G4 (American Type Culture Collection). Various numbers of spleen cells were added to 1×10^5 D10.G4 cells in 200 μl of culture medium, and [^{3}H]thymidine incorporation was measured 72 hr later (25).

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Abbreviations: APC, antigen-presenting cell; MHC, major histocompatibility complex.

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Treatment Protocols. Five to 8 days before islet transplantation, diabetic mice were injected once via the tail vein with 40–88 × 10^6 allogeneic, elutriated small lymphocytes or erythrocyte-depleted unfractionated spleen cells. Beginning on the day of lymphocyte injection, certain mice received 250 μg of the MR1 hamster anti-CD40L monoclonal antibody (19) intraperitoneally twice weekly for 2–7 wk or until graft failure.

RESULTS

Effect of Pretreatment with Allogeneic Lymphocytes on Islet Allograft Survival. We first measured survival of BALB/c (*H-2*^{d}) islet grafts in diabetic C57BL/6 (*H-2*^{b}) mice, some of which were transfused with (BALB/c × C57BL/6)F_1 small splenic lymphocytes. F_1 lymphocytes were used to avoid graft vs. host reactions. Because the ability of donor lymphocytes to tolerize could depend on their APC activity, this function was determined for each injected cell population. Fraction 19 was devoid of radioresistant APC function as documented by its inability to simulate proliferation of an alloreactive Th2 cell line; fraction 20 was nearly indistinguishable in size but had low APC activity (data not shown). Because the APC activity of activated B cells and other splenic APC is relatively radioresistant, these data suggest that fraction 19 was depleted of cells with constitutive APC activity (27, 28).

Diabetic mice given only islets rejected allografts within 13 days (9±2 days; range, 5–13 days; n = 23; Fig. 1). Mice transfused with higher doses of fraction 19 lymphocytes (75–88 × 10^6) showed longer graft survival (15±5 days; range, 7–21 days; n = 13), but the effect was temporary, and all islet grafts were rejected by day 21. Animals treated with smaller numbers of fraction 19 lymphocytes (40–44 × 10^6), with fraction 20 lymphocytes (75–88 × 10^6), or with unfractionated spleen cells rejected grafts with the same kinetics as did untreated recipients.

Pretreatment with Allogeneic Small Lymphocytes in Combination with Anti-CD40L. We expected allogeneic small lymphocytes to induce graft tolerance. We hypothesized that their failure to do so resulted from host T cells inducing or enhancing expression of costimulatory signals in the transfused leukocytes. To test this, we prevented the interaction of CD40L on host T cells with CD40 on donor leukocytes using an antibody against CD40L (19). Antibody was used in combination with allogeneic small lymphocytes to allow the presentation of alloantigen while preventing T cells from inducing costimulatory activity.

Again, all untreated recipients rejected islet allografts (n = 22; Fig. 2*A*). Treatment with anti-CD40L antibody by itself produced indefinite graft survival in 40% of recipient animals (n = 25; Fig. 2*B*) and delayed rejection in the remaining recipients (mean failure at 24±30 days; range, 10–125 days). This result suggests that anti-CD40L may modulate islet cell graft rejection in the absence of transfused allogeneic lymphocytes.

When anti-CD40L was injected in combination with allogeneic lymphocytes, we observed 96% long-term survival of islet grafts (n = 23), the one failure occurring on day 118 (Fig. 3*A*).

In additional experiments, 5 of 8 mice given one injection of small lymphocytes plus four injections of anti-CD40L antibody over 2 wk retained their grafts for the duration of the 6-wk experiment. Mice receiving unfractionated spleen cells and anti-CD40L antibody for 2 wk (n = 6) or 7 wk (n = 5) were similar in outcome; 2 mice in each group rejected grafts within 40 days. These results suggest that shorter courses of anti-CD40L antibody combined with small lymphocytes promote islet graft survival and that fractionated small lymphocytes may be more effective than unfractionated spleen cells.

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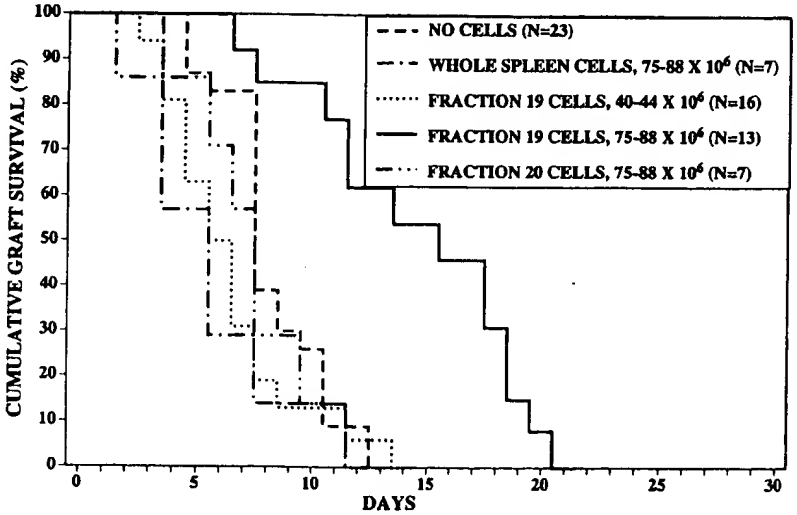

FIG. 1. Islet allograft survival (defined as plasma glucose <13.9 mM). Streptozotocin diabetic recipients were transfused with F_1 allogeneic spleen cells or small lymphocyte fractions of F_1 allogeneic spleen cells bearing islet donor alloantigens 5–8 days before transplantation.

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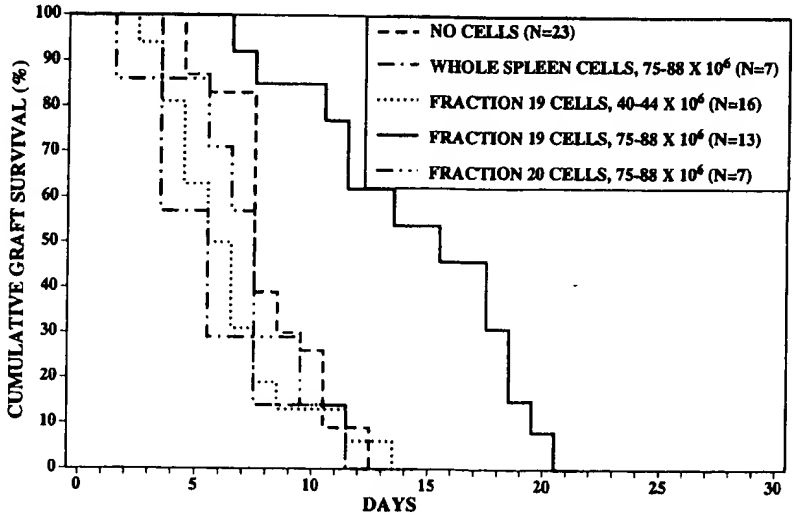

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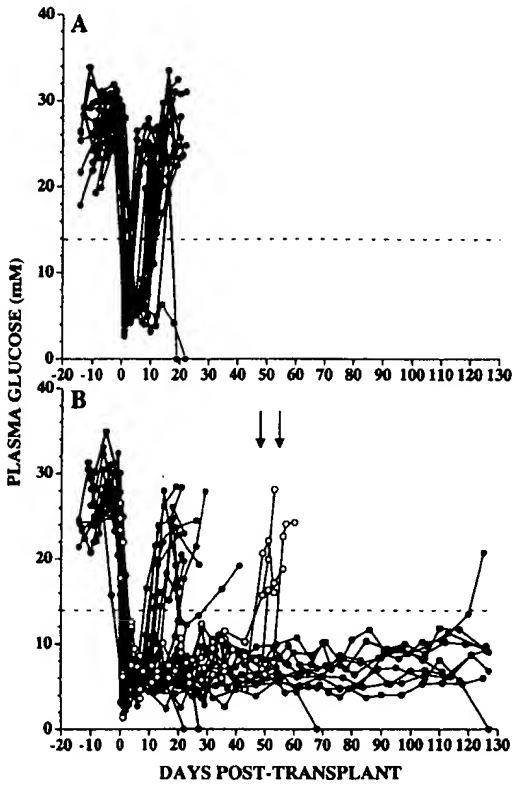


FIG. 2. Anti-CD40L by itself enhances islet allograft survival. Transplantation was done on day 0, and blood glucose of individual mice is shown. (*A*) Rejection of islet allografts in untreated control animals (n = 22, a separate control group from that shown in Fig. 1). (*B*) Animals treated with 250 μg of anti-CD40L antibody twice weekly for 1 wk before and 6 wk after transplantation (n = 25). O, Mice from which functioning islet allografts were removed by unilateral nephrectomy to confirm allograft survival on the days indicated by arrows (n = 4). Normoglycemic animals dead of unknown cause are shown as dropping to 0 mM glucose.

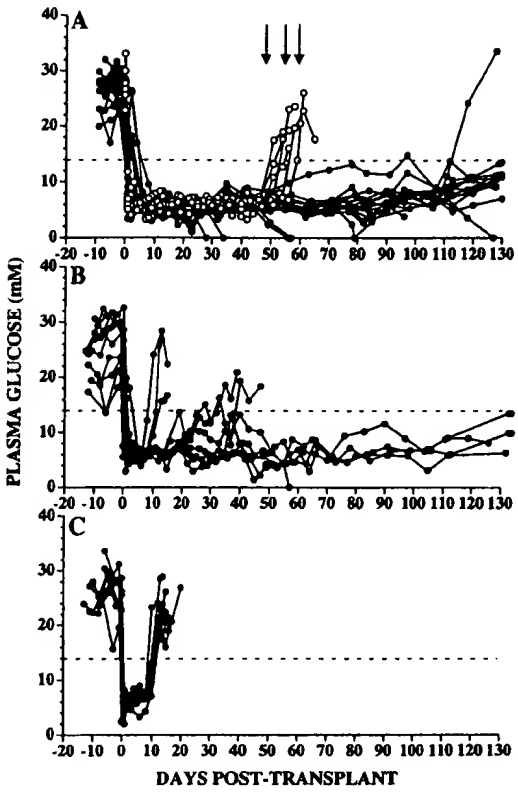


FIG. 3. Islet allograft survival. (*A*) Animals injected with 75–88 × 10^6 small lymphocytes (fraction 19) from (BALB/c × C57BL/6)F_1 mice 5–8 days before transplantation in combination with anti-CD40L antibody twice weekly for 7 wk beginning on the day of lymphocyte injection (n = 17). O, Mice from which functioning islet allografts were removed by unilateral nephrectomy to confirm allograft survival on the days indicated by arrows (n = 6). (*B*) Animals injected with (C57BL/6 × C3H)F_1 small lymphocytes (fraction 19) lacking BALB/c islet donor antigens in combination with anti-CD40L (n = 9). (*C*) Animals injected with (C57BL/6 × C3H)F_1 small lymphocytes (fraction 19) lacking BALB/c islet donor antigens without anti-CD40L (n = 8).

Fig. 3*B*). Transfusion of (C57BL/6 × C3H)F_1 lymphocytes without coadministration of anti-CD40L resulted in rapid rejection of BALB/c islets in all C57BL/6 recipients (Fig. 3*C*).

T Cells Are Not Required in the Spleen Cell Inoculum for Graft Survival. The small lymphocyte fraction includes T cells that might act as "veto" (29, 30) or suppressor cells. However, T-cell-depleted F_1 small lymphocytes were as effective as complement-treated control F_1 small lymphocytes for inducing allograft survival in combination with anti-CD40L (Fig. 4).

Recipient Antigens on Transfused Lymphocytes Are Not Required. Some models of tolerance induction by donor-specific transfusion require donor and recipient antigens on the same tolerizing cell (31, 32). To test this requirement, we next studied fully allogeneic lymphocytes, avoiding graft vs. host reactivity by T-depleting the donor inoculum. T-depleted, allogeneic lymphocytes in combination with anti-CD40L resulted in long-term graft survival (one failure at 128 days; Fig. 5*A*). These results were comparable to those obtained using F_1 lymphocytes (Fig. 4). In contrast, T-depleted allogeneic lymphocytes without anti-CD40L resulted in rapid rejection of islet allografts in all recipients (Fig. 5*B*).

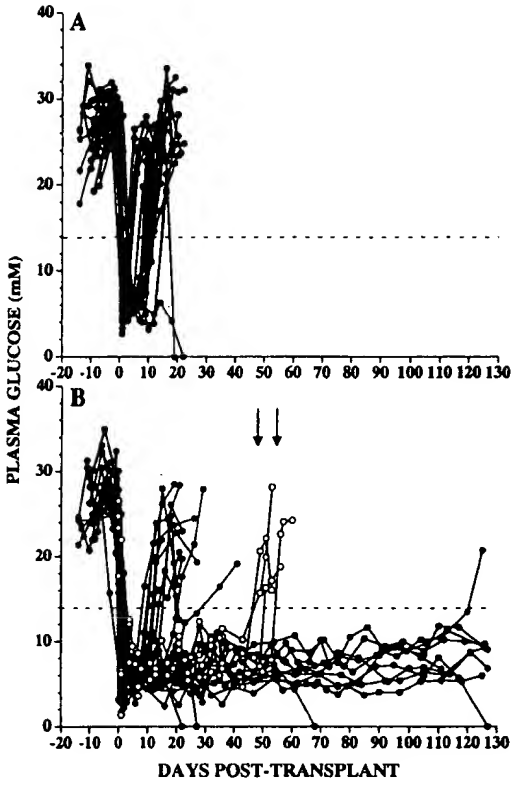
Survival of Islet Allografts after Discontinuation of Anti-CD40L Injections. To determine if anti-CD40L acts as a transiently immunosuppressive agent, mice were monitored after antibody was discontinued (Figs. 2–5). In all groups, with or without allogeneic lymphocyte injections, most animals whose grafts were functional when antibody was stopped remained normoglycemic thereafter.

Confirmation of Islet Allograft Function. Because all animals that became nondiabetic after transplantation gained weight, decreased metabolic demands cannot explain the observed normoglycemia. To confirm islet graft function and the absence of insulin secretion by residual native islets, the kidneys bearing islet implants were removed after 6–8 wk from 10 normoglycemic animals (Figs. 2*B* and 3*A*, O). In every case, removal of the grafted islets resulted in diabetes recurrence (3.3 ± 1.9 days; range, 2–8 days).

Histology of Transplanted Islets. Islet allografts were studied histologically in these 10 animals and in 58 instances of graft failure. BALB/c islet allografts from normoglycemic mice given F_1 small lymphocytes and anti-CD40L antibody appeared intact, showed no mononuclear infiltration, and contained well-granulated insulin- and glucagon-positive cells (data not shown). Graft sites from all 58 hyperglycemic animals evidenced either no residual islet tissue or only small remnants of islet tissue heavily infiltrated by mononuclear cells. In selected cases we performed immunohistochemical analyses for insulin, and none was detected.

DISCUSSION

Combined treatment with allogeneic small lymphocytes and a blocking antibody to CD40L allows permanent islet allograft survival between mouse strains that differ in major histocom-



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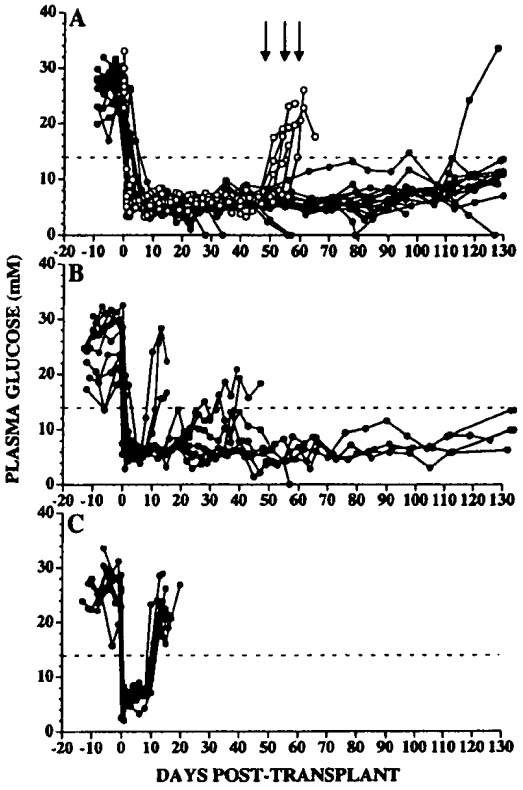

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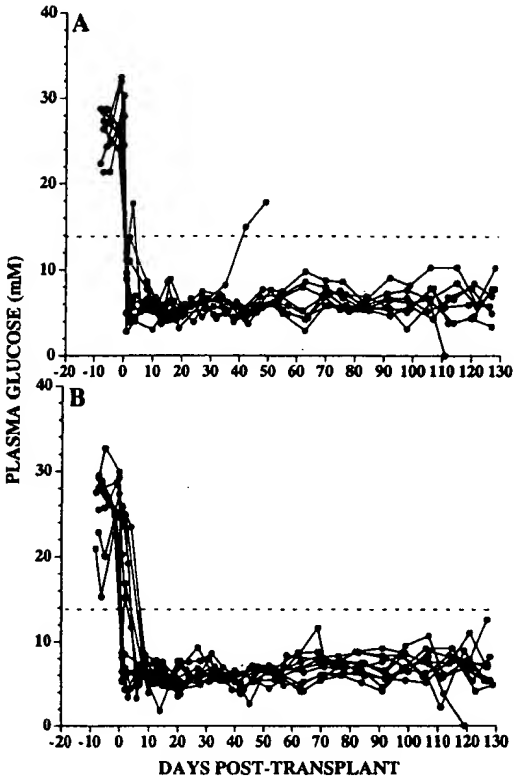


FIG. 4. T-cell-depleted F_1 small lymphocytes are effective in combination with anti-CD40L. (*A*) Animals injected with anti-CD40L and (BALB/c × C57BL/6)F_1 complement-treated small lymphocytes (n = 8). (*B*) Animals treated with anti-CD40L and (BALB/c × C57BL/6)F_1 small lymphocytes treated with anti-T-cell antibodies and complement (n = 10).

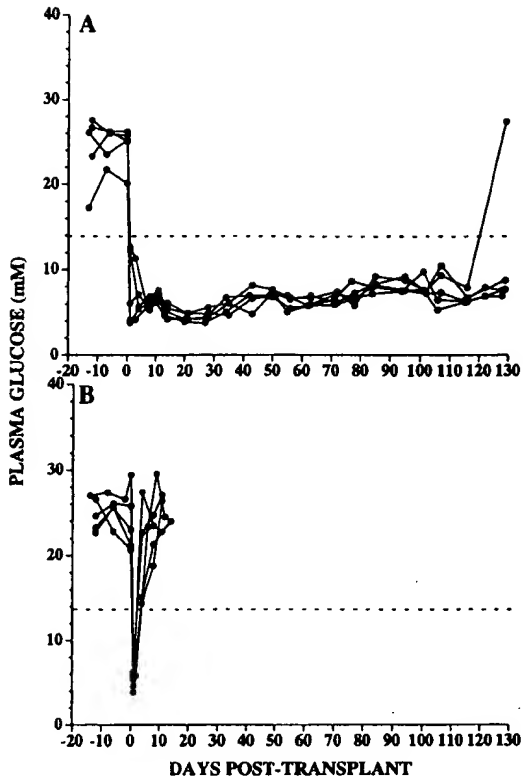


FIG. 5. Fully allogeneic small lymphocytes permit islet allograft survival in combination with anti-CD40L. (*A*) Animals treated with anti-CD40L and T-depleted BALB/c small lymphocytes (n = 5). (*B*) Animals treated with T-depleted BALB/c small lymphocytes without anti-CD40L (n = 5).

patibility complex (MHC)-encoded and minor transplantation antigens. Of 40 treated mice, 37 had permanent islet allograft survival, and 3 displayed delayed rejection (days 50, 127, and 130). In contrast, grafts were promptly rejected in all untreated animals. There was no evidence of graft rejection in the groups given anti-CD40L antibody and small lymphocytes after anti-CD40L was discontinued.

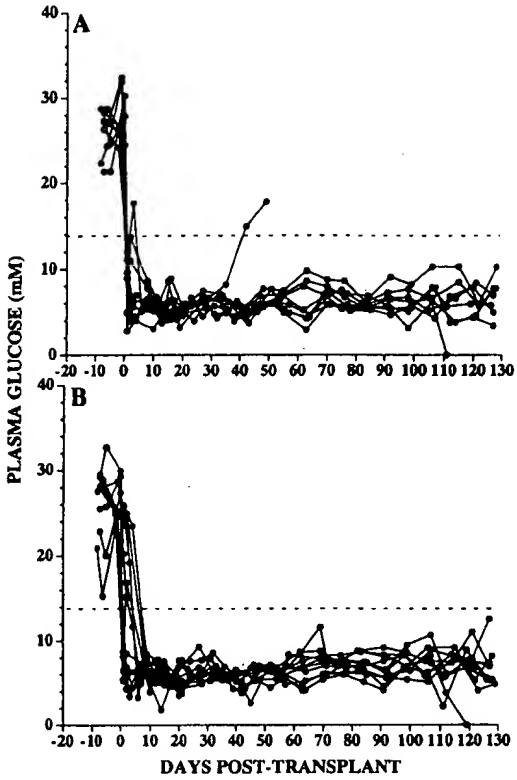
The effect of allogeneic small lymphocytes was donor antigen-specific. Small lymphocytes from F_1 animals of the donor and recipient strains and fully allogeneic, T-depleted small lymphocytes of the donor strain were equally effective when used in combination with anti-CD40L antibody. This suggests that non-T cells (B cells) in the donor splenocytes permitted permanent islet allograft survival when administered in combination with anti-CD40L antibody. In support of this hypothesis, we have recently shown that treatment with the anti-CD40L antibody prevents development of alloreactive cytolytic T lymphocytes after injection of allogeneic T-depleted spleen cells and induces specific tolerance to those alloantigens as measured by subsequent mixed lymphocyte responses *in vitro* (33).

We did not test for the nonspecific effects of hamster immunoglobulin on islet graft survival in these experiments, but in other studies nonspecific immunoglobulin had no effect on islet graft survival (unpublished data). Additionally, hamster immunoglobulin has no immunosuppressive effects on other T-dependent immune responses (34, 35), despite reports that it accelerates collagen-induced arthritis (36).

How do small allogeneic lymphocytes plus anti-CD40L enhance graft survival? Anti-CD40L may have a direct, inactivating effect on T cells responding to injected lymphocytes and allograft. CD40L is expressed on activated but not resting T lymphocytes (19, 37, 38). Injected allogeneic cells would induce CD40L expression on alloreactive T cells, rendering them susceptible to anti-CD40L. We think this is unlikely. (*i*) The antibody is not cytotoxic and does not clear $CD40L^+$ cytokine-producing cells *in vivo* (39). (*ii*) Although it blocks primary antibody responses, primed T cells can be recovered from animals immunized in the presence of anti-CD40L (34). (*iii*) As reported here, unfractionated spleen cells were nearly as effective as small lymphocytes in blocking graft rejection when combined with anti-CD40L. Unfractionated spleen cells would be expected to be more effective at inducing CD40L on alloreactive T cells, rendering them more susceptible to anti-CD40L antibody elimination.

We hypothesize that anti-CD40L blocks interaction of CD40L on alloreactive T cells with CD40 on donor leukocytes, preventing the induction or enhanced expression of costimulatory signals on those cells. The host T cells require CD40-dependent costimulatory signals to proliferate and differentiate into effector cells capable of graft rejection or at least to avoid inactivation as a result of antigen recognition on small B lymphocytes that lack costimulatory activity. To test this hypothesis, a disrupted CD40 gene (40) must be bred onto BALB/c mice to study CD40/CD40L interactions in our transplantation model.

The partial but substantial effect of anti-CD40L by itself on islet graft survival suggests that CD40L/CD40 interactions between donor islets and the host immune system may be important. Recent data show that dendritic cells (a passenger leukocyte in islet grafts) can be activated through CD40 (7). Alternatively, because CD40 is also on T cells (41) and macrophages (42), and induces secretion of chemotactic cytokines from monocytes and dendritic cells (7), CD40L/CD40



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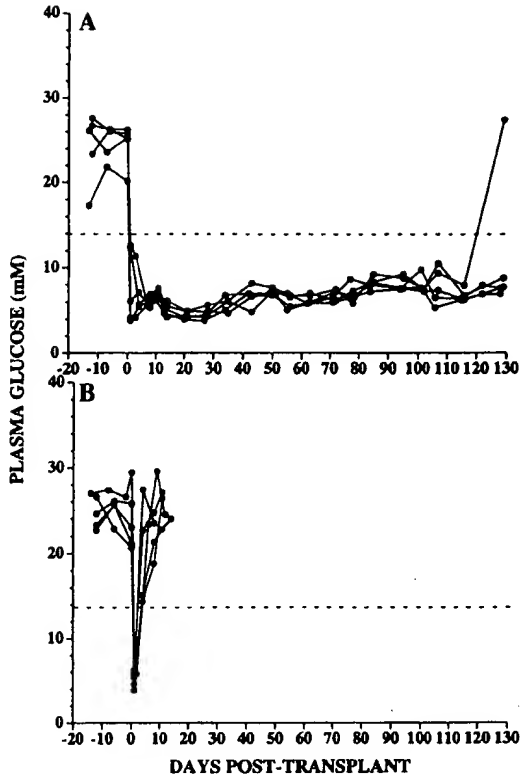

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interactions could be important in inflammation and graft destruction by alloreactive effector T cells.

Our limited success in prolonging graft survival across a complete MHC mismatch with small lymphocytes alone contrasts with earlier reports. Skin graft survival has been observed across an isolated class II difference (43) and the male minor transplantation antigen (44) by pretreatment with APC-depleted spleen cells or purified B cells, respectively. We have also been able to induce tolerance to a transgene-encoded antigen expressed in B cells by transferring transgenic B cells into nontransgenic littermates without anti-CD40L (V. Yuschenkoff and D.C.P., unpublished). The difference between those results and our observations here may be due to the high frequency of alloreactive T cells in complete MHC mismatches. Some of these alloreactive T cells may be primed or recently activated by crossreacting environmental antigens (45) and therefore difficult to tolerize (44). They may also be more effective than naive T cells in inducing costimulatory activity and converting transfused lymphocytes into immunogenic APCs. Others have reported tolerance induction across MHC barriers by injecting spleen cells without removing nonlymphoid APCs (32, 46), but we find this possible only if CD40L/CD40 interaction is blocked.

We thank Linda Paquin, Linda Leehy, Olita Treimanis, and Elaine Norowski. This work was supported in part by a Diabetes Interdisciplinary Research Program grant from the Juvenile Diabetes Foundation (A.A.R.), National Institutes of Health Grants DK41235 (J.P.M.), DK25306 (A.A.R.), DK36024 (D.L.G.), and AI26296 (R.J.N.), and grants from the Arthritis Foundation and the Multiple Sclerosis Society (R.J.N.).

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interactions could be important in inflammation and graft destruction by alloreactive effector T cells.

Our limited success in prolonging graft survival across a complete MHC mismatch with small lymphocytes alone contrasts with earlier reports. Skin graft survival has been observed across an isolated class II difference (43) and the male minor transplantation antigen (44) by pretreatment with APC-depleted spleen cells or purified B cells, respectively. We have also been able to induce tolerance to a transgene-encoded antigen expressed in B cells by transferring transgenic B cells into nontransgenic littermates without anti-CD40L (V. Yuschenkoff and D.C.P., unpublished). The difference between those results and our observations here may be due to the high frequency of alloreactive T cells in complete MHC mismatches. Some of these alloreactive T cells may be primed or recently activated by crossreacting environmental antigens (45) and therefore difficult to tolerize (44). They may also be more effective than naive T cells in inducing costimulatory activity and converting transfused lymphocytes into immunogenic APCs. Others have reported tolerance induction across MHC barriers by injecting spleen cells without removing nonlymphoid APCs (32, 46), but we find this possible only if CD40L/CD40 interaction is blocked.

We thank Linda Paquin, Linda Leehy, Olita Treimanis, and Elaine Norowski. This work was supported in part by a Diabetes Interdisciplinary Research Program grant from the Juvenile Diabetes Foundation (A.A.R.), National Institutes of Health Grants DK41235 (J.P.M.), DK25306 (A.A.R.), DK36024 (D.L.G.), and AI26296 (R.J.N.), and grants from the Arthritis Foundation and the Multiple Sclerosis Society (R.J.N.).

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How B and T cells talk to each other

Edward A. Clark & Jeffrey A. Ledbetter

The B cells of the immune system, which secrete antibodies against foreign antigens, are fully specific and effective only after maturation in lymph nodes and other lymphoid tissues. Immunocompetent T cells play a crucial part in this process, but the molecular details of the way in which the two cell types interact have only recently become apparent.

MILLIONS of newly formed lymphocytes leave the bone marrow daily[1]. Each of these cells either multiplies and differentiates or dies, depending on the nature, timing and location of interactions with other cells of the immune system[2,3]. Specific recognition of foreign antigen by cell-surface immunoglobulin (Ig) induces B cells to proliferate and differentiate either into plasma cells, which produce soluble immunoglobulin to fight infection, or into memory B cells which can respond rapidly to subsequent encounters with the same antigen.

This process, however, requires help from activated T cells. Antigen-specific T-cell activation is dependent on interactions between T cells and specialized antigen-presenting cells, which can themselves be B cells. Once active, the T cells in turn promote B-cell activation, both by releasing T-cell-derived cytokines such as interleukin (IL) -2, -4 and -5, and by direct intercellular contact[2-6]. What follows is an account of some of the molecules responsible for the second part of this process.

Germinal centre formation

Resting B cells circulate in the blood, migrating across high endothelial venules to sites of trapped antigen in secondary lymphoid organs such as lymph nodes, the spleen, tonsils and Peyer's patches (Fig. 1*a*). B cells bearing immunoglobulin specific for trapped antigen enter the T-cell-rich paracortical regions below the outer layer, or cortex, of lymphoid tissues[2,4,5], where they capture and process the antigen[7]. Some T cells in this area may already have been activated by recognizing antigen fragments bound to the major histocompatibility complex (MHC) class II on antigen-presenting cells (Fig. 1*b*, *c*). Once activated, the cell-surface receptors of the T cell and the cytokines it releases induce antigen-bearing B cells to migrate into B-cell follicles; other types of antigen-presenting cells, such as the bone-marrow derived dendritic cell, may also fulfil this function.

In the follicle, B cells interact with follicular dendritic cells. Unlike dendritic cells, these large spiny cells are not derived from the bone marrow; they specialize in binding antibody–antigen immune complexes and in inducing B-cell proliferation and differentiation. This interaction leads to the formation of a finely structured germinal centre (Fig. 1*c*). Initially, rapid B-cell proliferation gives rise to a dark zone, relatively devoid of T cells (Fig. 1*d*). Maturing B cells then move into the light zone, which contains abundant follicular dendritic cells and some T cells. It is here, probably within the basal light zone, that the variable (*V*) region immunoglobulin genes of B cells undergo somatic mutation to increase their affinity for antigen[5]. This process occurs within days of antigenic stimulation[8,9], and only during germinal-centre development[5]. B cells with high-affinity receptors for antigen are subsequently 'selected', and switch from producing IgM to another immunoglobulin class. This step requires activated B cells, CD4-positive T cells and follicular dendritic cells (Fig. 1*e*). Eventually, germinal-centre B cells mature into either memory or plasma B cells, perhaps in response to signals in the apical light zone, which is rich in a subtype of follicular dendritic cell[2,4].

Signals that 'talk'

A number of the receptor–coreceptor pairs mediating the effects of T cells on B-cell maturation are very well characterized (Fig. 2). In the paracortical region of lymphoid tissue (Fig. 1*c*), bone-marrow derived dendritic cells are very effective at presenting MHC class II-bound peptides to $CD4^+$ T cells, as they express high levels of MHC class II and CD40, as well as key accessory molecules such as CD54, CD58 and CD80 (formerly termed B7 or BB1). Although the affinity of T-cell receptors for MHC class II-bound peptide is low[10], the CD4 and CD2 coreceptors on T cells help signalling through the receptor after binding to MHC class II and CD58 (LFA-3), their respective ligands on the bone-marrow derived dendritic cells. Crosslinking of the T-cell receptor and its associated coreceptors in turn leads to the rapid activation of the CD11a/18 (LFA-1) complex[11], which can then bind to ligands such as CD54. The fact that bone-marrow derived dendritic cells express CD54 as well as CD58 and CD80 (Fig. 1*b*) may explain why naive T cells can be activated by these dendritic cells but not by resting B cells, which lack these receptors[12,13]. Once B cells have been activated first by T cells (Fig. 1*c*) and later by follicular dendritic cells (Fig. 1*e*), they do express an array of surface receptors (Fig. 1*e*) and can present antigen to T cells effectively.

Cell-surface adhesion molecules, such as integrins (for example CD11a/18), selectins and CD58/59, are all broadly distributed, and are likely to affect the migration and adhesion of lymphocytes in general and to modulate lineage-specific signals. Other molecules are relatively restricted to antigen-presenting cells, T cells or B cells, and must therefore function in processes unique to lymphocyte maturation (Fig. 2). Two such molecules, CD40 and CD80, which are neither integrins nor selectins, bind coreceptors whose levels are affected by the state of the T cell expressing them (Fig. 2). These receptor–ligand pairs allow T and B cells to sense the state of their partner's activation.

CD40 and its ligand

CD40, a surface glycoprotein of 45–50K relative molecular mass, is related to the receptors for tumour necrosis factor (TNF)-α and is expressed on late pre-B cells in bone marrow, mature B cells and certain accessory cells, including bone-marrow derived dendritic cells and follicular dendritic cells (Fig. 1). Crosslinking CD40 promotes B-cell proliferation[6,14], prevents apoptosis of germinal-centre B cells[4], and promotes immunoglobulin class switching[15]. The ligand for CD40, CD40L (also called gp39, 5c8 or TRAP), is a TNF family member expressed on activated but not resting T cells[16,17]. When the CD40–CD40L interaction is blocked *in vitro* with soluble CD40 or monoclonal antibodies to CD40L (gp39), B cells cannot proliferate or produce immunoglobulin in response to T-cell signals (refs 16–19, and see ref. 20 for review), indicating that the interaction is required for signalling. Resting T cells that lack CD40L cannot help B cells.

Recently, the gene encoding CD40L has been shown to be defective in patients with hyper-IgM (HIM) syndrome[21]. Although these patients have IgM-producing B cells, they do not form germinal centres in response to foreign antigen. Their B cells are capable of switching from IgM to IgG or IgE production *in vitro* when exposed to IL-4 and monoclonal antibodies against CD40, but they do not switch immunoglobulin classes *in vivo*. T cells and CD40L–CD40 interactions are thus required

for both germinal-centre formation and immunoglobulin class switching, but not for at least some IgM responses.

As such interactions can theoretically take place either in T-cell zones (Fig. 1*c*) or in basal light zones (Fig. 1*e*), it is not yet clear where the CD40L–CD40 interactions necessary for germinal-centre formation or class switching take place. Interestingly, patients with HIM syndrome are also prone to infections with pathogens such as *Pneumocystis carinii*, prevalent in T-cell deficiencies such as AIDS[22]. Antibodies against CD40L block the development of both primary and secondary antibody responses[23] and of collagen-induced arthritis[24], confirming the key role of this interaction in B-cell regulation.

CD40 is expressed on follicular dendritic cells, bone-marrow derived dendritic cells, activated macrophages and thymic epithelial cells; CD40–CD40L interactions may thus regulate T-cell maturation via interactions with non-B cells. Crosslinking CD40 on some non-B cells has indeed been shown to augment cytokine production (see, for example, ref. 25).

The CD80 family and CD28/CTLA-4

Whereas the CD40L–CD40 interaction enables the B cell to respond to an activated T cell, the interaction between CD80 and CD28 allows peripheral T cells to respond to an activated B cell by dividing and producing cytokines required for T-cell differentiation. CD80 was originally defined as a B-cell activation marker[26,27], but it is now known to be one of a family of related molecules that share the same receptor and are found on B cells, activated macrophages and some bone-marrow derived dendritic cells[28,29]. Of the ligands for the CD80 family, CD28 (ref. 30) is found on both resting and activated T cells, whereas the other, CTLA-4 (ref. 31), is found only on activated T cells. The binding of CD80 to CD28 on T cells previously stimulated through their antigen receptors increases IL-2 production and T-cell proliferation[32].

The CD28 signal is discussed in detail elsewhere[33]. Interference with this signal *in vitro* can block T-cell proliferation[34] and B-cell maturation induced by T-cell cytokines[35]. A soluble form of CTLA-4 (CTLA4Ig) interferes both with CD80-dependent signalling to T cells *in vitro*[31] and with primary antibody responses in mice, demonstrating the importance of CD80 in B-cell maturation[36]. By contrast, weakly immunogenic tumours transfected with B7 complementary DNA induce a protective T-cell response[37,38], showing that CD80 expression can dictate whether or not cellular antigens are 'recognized' by the immune system.

A reciprocal dialogue

When and where might the CD28–CD80 interaction occur? CD80 expression is induced or stimulated after crosslinking MHC class II with monoclonal antibodies[34], during allogeneic T-cell interactions with bone-marrow derived dendritic cells[39], and during autoreactive T-cell interactions with B cells[40]. Interactions with T cells which crosslink MHC class II may thus induce CD80 expression in B cells so that CD80 can, in turn, signal to the T cell via CD28. Induction of CD80 expression indeed requires the cytoplasmic tail of MHC class II[40].

CD80 expression may therefore coincide with CD40L expression on T cells responding to MHC class II-bound peptides (Fig.

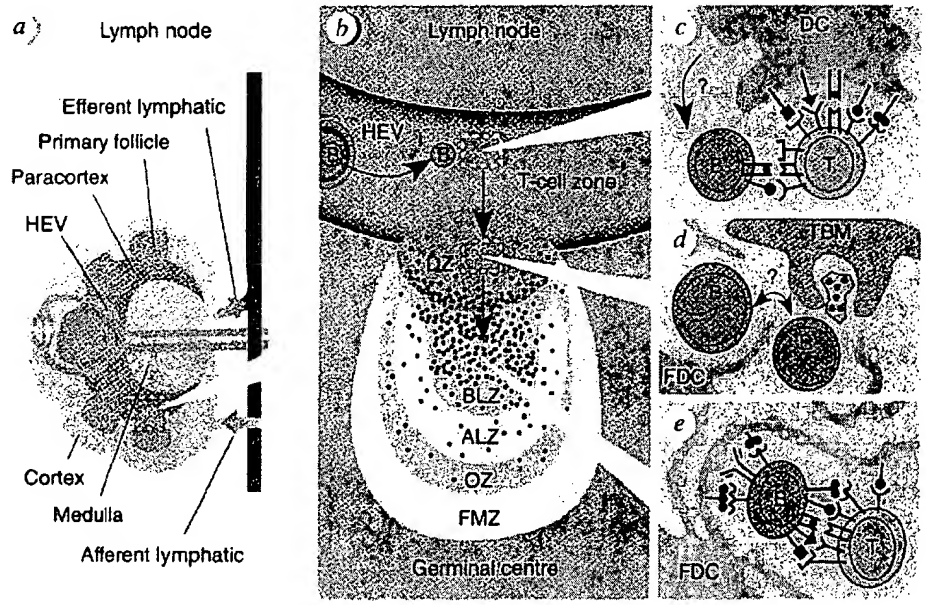

FIG. 1 Journey of a naive antigen-specific B cell during the development of a primary T-cell-dependent response (after refs 2 and 4). *a*, The newly-formed circulating B cell enters the lymph node via high endothelial venules (HEV). *b*, The antigen-specific B cell migrates to a paracortical T-cell zone where dendritic cells (DC) have processed and presented antigen to CD4⁺ T cells. *c*, Dendritic cells are very effective antigen-presenting cells (APC), in part because they express high levels of class II (Ψ) and CD40 (●), and unlike naive B cells, they express CD54 (●●), CD58 (▼) and CD80 (■) which are ligands for CD11a/18 (Ψ), CD2 (Y) and CD28 (Ψ) on T cells. One probable sequence may be that T cells via their T-cell receptor/CD3 complex (M) and CD28 recognize class II + peptide as well as CD80 on tissue dendritic cells and are efficiently activated to express activated CD11a/18, CD40L (Ψ) and cytokines which in turn contribute to the activation of B cells. Unidentified signals induce a small number of activated B cells to migrate to a follicle where they begin to proliferate. *d*, Activated B cells become localized in the follicle, associate loosely with a network of follicular dendritic cells (FDC), and begin a rapid and oligoclonal proliferation followed by hyper-somatic mutation of B-cell immunoglobulin V-region genes, which leads to expansion to up to 10,000 B cells in 3 days; as a result, a dark zone (DZ) containing B centroblasts and tingible body macrophages (TBM) (*d*) and a light zone (LZ) containing a dense FDC network and centrocytes (derived from centroblasts) (*e*) are formed. *d*, Within the basal light zone (BLZ), some centrocytes with high-affinity IgM receptors (Ψ) are stimulated by specific antigen retained on CD54⁺ follicular dendritic cells and are selected to survive after crosslinking of surface IgM or CD40 (ref. 2) while other centrocytes not selected undergo apoptosis and fall back in the dark zone where they are devoured by tingible body macrophages. The interaction between activated B cells, which express a full battery of cell–cell interaction molecules, and follicular dendritic cells may require CD49*a* (VLA-4) (Ψ) and CD106 (VCAM-1) (●●●) and CD11a/18 and CD54. Selected B cells may process and present antigen to CD4⁺ CD40L⁺ T cells, which can be found in the basal light zone[43]; these T cells in turn may induce the B cells to undergo immunoglobulin class switching, to survive and to proliferate as memory B cells, processes involving CD40 crosslinking, IL-4, IL-10 and other cytokines. In the apical light zone (ALZ), B cells interact with CD23⁺ follicular dendritic cells and receive signals via IL-1, CD23 or other surface receptors, and thereby may be induced to become plasma cells or to become memory B cells. OZ, Outer zone; FMZ, follicular mangle zone.

3). Antibodies to MHC class II and CD3 indeed induce expression of CD80 and CD40L, respectively, with similar kinetics[19,34]. Crosslinking CD28 on activated T cells also increases the expression of CD40L (S. Klaus *et al.*, manuscript in preparation), and crosslinking CD40 on B cells increases the expression of CD80 (ref. 41) and B70/B7-2 (ref. 28). This reciprocal dialogue may occur in peripheral lymphoid T-cell zones after T-cell activation, where CD40L$^+$ T cells have been identified in both mice and humans[42,43], or in the basal light zones of germinal centres in humans, where CD40L$^+$ T cells have also been found[43].

Controlling the dialogue

Interactions between T and B cells must be carefully regulated in order to prevent activation of self-reactive or bystander cells. The presence of CD40 and MHC class II on resting B cells, or the T-cell receptor/CD3 complex and CD28 on resting T cells, is insufficient to trigger mutual T–B activation[3,12,13]. Indeed, rather than activating resting T cells, resting B cells tolerize them to specific antigens[3]. A reciprocal dialogue can ensue, however, if either the T or B cells are activated (Fig. 3). Activated T cells expressing CD40L induce resting B cells to express CD80 (ref. 41) and activated B cells expressing CD80 induce T cells to express CD40L (S. Klaus *et al.*, unpublished data). Signalling through CD40 on B cells or CD28 on T cells is most efficient after stimulation through their respective antigen receptors[6,33], ensuring that the interaction is specific.

Remarkably, the signals delivered by CD40 and CD28 have similar effects. Both promote cell proliferation stimulated by antigen receptor crosslinking[6,14,33]. Moreover, crosslinking of CD28 prevents specific unresponsiveness or apoptosis that would otherwise occur on stimulation of the T-cell receptor[44,45]. Similarly, signals delivered by CD40 on contact with T cells can block apoptosis of immature B cells induced by crosslinking their surface immunoglobulin receptors[46].

The CD40L–CD40 and CD28/CTLA-4–CD80 ligand pairs (Fig. 3) are clearly not the only means by which T cells and B cells interact. The CD11a/18–CD54 ligand pair (Fig. 2) is also likely to play a part, as: (1) an active form of CD11a/18 is rapidly induced on crosslinking the T-cell receptor complex[11]; (2) antigen-specific T-cell activation rapidly induces a CD11a/18–CD54-dependent signal to the antigen-presenting B cell[47]; and (3) crosslinking CD40 on B cells promotes allogeneic T-cell proliferation via CD11a/18–CD54-dependent interactions[48]. Patients with leukocyte adhesion defect do not express CD11a/18 on their T or B cells. They make both IgM and IgG antibodies in response to specific antigen, but have depressed antibody titres, indicating that their production of memory B cells may be impaired[49]. The CD11a/18 receptor may therefore contribute to T-cell-dependent B-cell maturation, but is not as essential as CD40L[3,21].

Surface IgM and associated adhesion molecules

Cell-surface immunoglobulin plays a critical role in several stages of B-cell maturation, including the pre-B-cell and immature B-cell stages in the bone marrow[50]. Crosslinking of cell-surface IgM by antigen-bearing follicular dendritic cells also prevents B cells in germinal centres from undergoing apoptosis[4]. A number of surface molecules and protein kinases are associated with cell-surface IgM[50]; how these molecules contribute to its signalling activity is unclear. Two B-cell-specific adhesion molecules, CD19 (ref. 51) and CD22 (ref. 52) (both members of the immunoglobulin supergene family), may associate with surface IgM and are rapidly phosphorylated on tyrosine residues after it becomes crosslinked[53,54]. CD22 may be required for normal signalling, as intracellular calcium is not elevated after crosslinking surface immunoglobulin in CD22-negative B cells (see ref. 54 for review). CD22 also interacts with several ligands on B and T cells containing α2,6-linked sialic acids[54], including the activation marker CD45RO, and is therefore likely to affect antigen-specific B-cell maturation.

CD19 is expressed on B cells throughout their maturation. Although its ligand has not yet been identified, it interacts with various molecules on other B cells, including the receptor for the C3d component of complement, CD21/CD2 (ref. 55), indicating that it may facilitate a complement-mediated signalling pathway. When crosslinked, CD19 induces calcium mobilization in both pre-B and mature B cells. After surface IgM becomes crosslinked, phosphorylated tyrosine residues in CD19 are capable of binding to the SH2 domains of proteins such as the tyrosine kinases c-abl and PI-3 kinase. CD19 indeed binds to the SH2 domains of PI-3 kinase after crosslinking of surface IgM[53,56]. It may therefore bring key signal-transducing enzymes to the plasma membrane.

Discussion

The CD28–CD80 and CD40–CD40L receptor–ligand pairs are essential for the dialogue between B and T cells. Both CD28- and CD80-deficient mice have normal levels of B and T cells and produce immunoglobulins of all classes[57,58], indicating that the immune system has evolved alternatives to the CD28–CD80 pathway; these are now known to include CTLA-4 (ref. 31) and B70/B7-2 (refs 28, 29). By contrast, CD40–CD40L has no back-up: individuals defective for CD40 (ref. 59) or CD40L[21] do not form germinal centres or switch immunoglobulins. Once CD40 and CD40L engage, CD40L is rapidly internalized[60], ensuring that this singular and potent interaction is short-lived.

Many questions about B-cell maturation remain. First, what signals other than CD40 are required for germinal-centre forma-

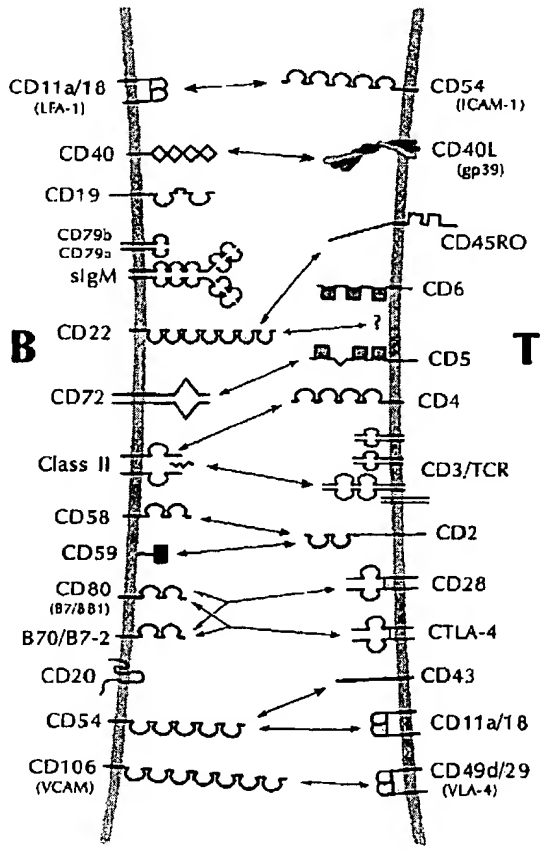

FIG. 2 Adhesion molecules that may mediate interactions between activated CD4$^+$ T cells and activated B cells. Domain structures are indicated as follows: immunoglobulin superfamily domains (ωωω), nerve growth factor receptor superfamily cysteine-rich domains in CD40 (◇◇◇), C-type lectin domains in CD72 (⌒⌒), scavenger receptor domains in CD5 and CD6 (▮▮▮), and tyrosine phosphatase domains of CD45 (⌒⌒).

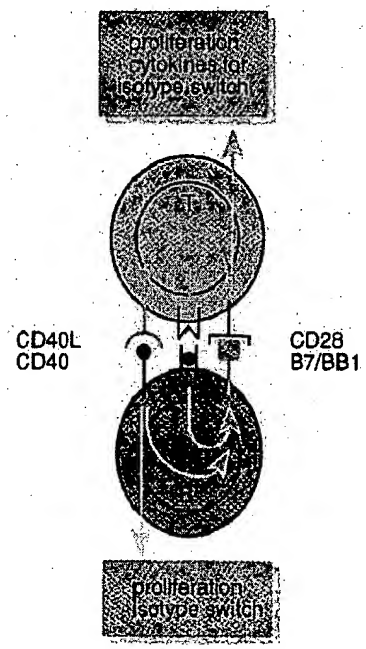

FIG. 3 A reciprocal dialogue between T cells and B cells during T-cell-dependent B-cell maturation. Recognition of class II + peptide by T cells leads to activation of the T cell and new expression of CD40L (Y) and also, via crosslinking of class II, B cells are activated leading to new expression of CD80(B7/BB1) (■). Engagement by CD40L of its receptor CD40 (●) on B cells and by CD80 of its receptor CD28 (Y) on T cells amplifies the initial cell activation and leads to B-cell or T-cell proliferation. The activated T cell is induced by CD28 crosslinking to make cytokines needed for immunoglobulin class switching and to express CD40L. The activated B cell is induced by CD40 crosslinking to prepare for cytokine-dependent immunoglobulin class switching and to express CD80 or related molecule B70/B7 (ref. 28). This reciprocal activation and maturation only occurs if either the T cell or the B cell have been previously stimulated to express CD40L or CD80, for example, via an encounter with antigen. Not shown are other receptors, particularly the CD11a/18 (LFA-1) integrin and its ligands such as CD54, which may function to stabilize and modulate the lineage-specific signalling.

tion? Second, what signals induce the somatic mutation of immunoglobulin variable-region genes in B cells, and the subsequent selection of high-affinity antibodies? In addition to their role as antigen depots, follicular dendritic cells also induce this process, while the germinal-centre signals preventing B-cell apoptosis may allow time for it to occur. It remains to be seen, however, what signals direct immunoglobulin class switching, or induce the switched B cells to become plasma cells or memory cells.

Despite this, several basic features of the T–B-cell dialogue have already emerged. First, it is likely that each receptor transmits a message to the cell. Second, other components of the intercellular signalling apparatus, such as cytokines, can affect the expression and activity of cell-surface receptors and, conversely, signalling by the receptors can alter cytokine expression. Finally, just as cytokines may provide different signals depending on their context, so cell-surface receptors may transmit different signals depending on the state of B-cell maturation. A complete understanding of the dialogue will thus require an analysis of the way in which the signals delivered by these components interact. □

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ACKNOWLEDGEMENTS. This work was supported by the NIH and by Bristol Myers Squibb Pharmaceutical Research Institute. We thank A. Akbar, R. Armitage, D. Fearon, T. Kipps, S. Lederman, R. Noelle and H. Ochs for permission to cite papers in press or unpublished data. We also thank S. Klaus and C.-L. Law for critical comments and N. Quense, K. Elias and M. Domenowske for drawing, editorial and graphics assistance.